Virological and Immunological Studies of Human Cytomegalovirus Infection in Allogeneic Stem Cell Transplant Recipients

A Thesis submitted to University College London, 2007

for examination for a PhD in Infection

By

Hubertus Carolus Eduard Buyck

MB ChB, FRACP, FRCPA

Centre for Virology,

Department of Infection,

Royal Free and University College Medical School,

Rowland Hill Street, London NW3 2PF

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I designed the studies described in Chapter Two and Three of this thesis. I personally collected all the data for Chapters Two and Three. The data entry and statistical analysis is all my own work. The real time PCR viral loads performed on retrospective samples were done by me, and the remainder of the real time PCR viral loads were performed by the laboratory staff of the Department of Virology at the Royal Free Hospital.

The data for Chapter Four and Five was generated from a collaborative study designed by myself and Dr. Samantha Paston at the Anthony Nolan Research Institute. I recruited all the patients and collected all the clinical data. Sample collection and mononuclear cell preparation was performed equally by both myself and Dr. Samantha Paston. The CD4+ T cell stimulation experiments were performed by me, while Dr. Samantha Paston performed the CD8+ T cell stimulation experiments. The FBC measurement of normal control samples was performed by me and the patient samples were performed by the laboratory staff at the Department of Haematology. The tissue typing was performed in the Tissue Typing Laboratory at the Anthony Nolan Research Institute. The data entry and statistical analyses are all my own work.

Professor V. Emery

Dr. Hubertus C. E. Buyck

Principle Supervisor

Date: 18.05.2007

Abstract

Human cytomegalovirus (HCMV) is the most common viral infection complicating stem cell transplantation, and if untreated frequently results in a fatal outcome. In order to identify the main risk factors for HCMV infection, a retrospective study of all allogeneic stem cell transplants performed over a five year period at a single centre was undertaken. The main risk factors for HCMV infection following transplantation were identified as a HCMV seropositive donor and/or recipient and in-vivo use of the monoclonal antibody, anti-CD52 (alemtuzumab). A prospective study of HCMV viral loads determined by real time PCR using a Taqman probe was undertaken, and the viral load dynamics of 57 patients were analysed. Despite the use of aciclovir prophylaxis, PCR monitoring and pre-emptive therapy, the peak viral load, viral replication rate and the total duration of viraemia remain significant risk factors for symptomatic HCMV infection. Peak viral load was the most significant predictor of time to viral clearance. A prospective longtitudinal study of the reconstitution of the HCMV specific immune response following allogeneic stem cell transplantation was performed in 20 patients using intracellular interferon gamma staining and flow cytometry. HCMV infection was associated with a significantly reduced HCMV specific CD4+ T cell response. Furthermore, in patients receiving in-vivo anti-CD52, HCMV specific CD4+ T cell immune recovery was significantly delayed. An HLA class II epitope mapping study was undertaken in stem cell transplant recipients and healthy controls using peptide pools consisting of 15 mer overlapping

peptides spanning the entire amino acid sequence of the HCMV proteins, pp65 and IE1. Using intracellular cytokine detection in CD4+ T cells, a number of novel HCMV specific class II epitopes were identified. Transplant recipients responded to a broader range of epitopes than HCMV seropositive controls. These results have important implications for HCMV specific immunotherapy in allogeneic stem cell transplantation.

Table of Contents

Declaration 2
Abstract 3
Table of Contents 5
List of Tables 10
Table of Figures 12
Abbreviations 15
Definitions and Conventions 17
Acknowledgements 18

Chapter 1 Introduction 20
1.1 Allogeneic Stem Cell Transplantation 20
1.1.1 A Historical Perspective 20
1.1.2 Early Scientific Experiments 21
1.1.3 Bone Marrow Transplant Pioneers 21
1.1.4 Tissue Typing and Graft Versus Host Disease 22
1.1.5 Post Transplant Immunosuppression and Refinements of the Conditioning Regimen 24
1.1.6 T-cell Depletion 25
1.1.7 Unrelated Stem Cell Transplants 26
1.1.8 Alternative Donor Sources 27
1.1.9 Peripheral Blood Stem Cell Transplants 28
1.1.10 Reduced Intensity Conditioning Transplants 29
1.1.11 Donor Lymphocyte Infusion 30
1.2 Indications for Allogeneic Transplantation 30
1.2.1 Acute Myeloid Leukaemia and Myelodysplasia 31
1.2.2 Acute Lymphoblastic Leukaemia 32
1.2.3 Chronic Myeloid Leukaemia 33
1.2.4 Aplastic Anaemia 34
1.2.5 Thalassaemia and Sickle Cell Anaemia 34
1.2.6 Other Haematological Conditions 35
1.2.7 Congenital Immunodeficiency Syndromes and Other Non-Malignant Conditions 36
1.3 Complications of Transplantation 37
1.3.1 Infectious Complications 37
1.3.2 Non-infectious Complications 39
1.4 Discovery of Human Cytomegalovirus 42
1.5 Classification of Human Cytomegalovirus 43
1.6 Epidemiology of Human Cytomegalovirus 44
1.7 Human Cytomegalovirus Structure and Organisation 45
1.7.1 Structure 45

1.7.2 Genomic Organisation 46
1.7.3 Important Structural Proteins 49
1.8 Human Cytomegalovirus Infection 52
1.8.1 Tissue tropism 52
1.8.2 Viral Replication 52
1.8.3 Latency and Reactivation 56
1.9 Role of the Immune Response in Controlling Human Cytomegalovirus Infection 57
1.9.1 Innate Immune Response 58
1.9.2 Humoral Immune Response 59
1.9.3 Cytotoxic T Cell Response 59
1.9.4 Helper T Cell Response 61
1.10 Immune Manipulation by HCMV 64
1.11 Immune Recovery Following Allogeneic Stem Cell Transplantation . 70
1.12 Clinical Features of Human Cytomegalovirus Infection 73
1.12.1 Infection in the Immunocompetent 73
1.12.2 Infection in the Immunosuppressed Host 74
1.13 HCMV Infection in Allogeneic Stem Cell Transplantation 77
1.13.1 Disease Manifestations 78
1.13.2 Risk Factors 79
1.13.3 Indirect Effects 80
1.13.4 Impact of HCMV Serology on Transplant Mortality 81
1.14 Laboratory Diagnosis and Monitoring 82
1.14.1 Serology 82
1.14.2 Tissue Diagnosis 82
1.14.3 Cell Culture Techniques 83
1.14.4 pp65 Antigenaemia 84
1.14.5 Polymerase Chain Techniques 85
1.15 Antiviral Therapy 86
1.15.1 Aciclovir 86
1.15.2 Ganciclovir 87
1.15.3 Foscarnet 89
1.15.4 Cidofovir 89
1.15.5 Formivirsen 90
1.15.6 Combination Therapy 90
1.16 Strategies for the Prevention and Treatment of HCMV Disease in Allogeneic Stem Cell Transplantation 92
1.16.1 Prevention 92
1.16.2 Treatment of established disease 92
1.16.3 Intravenous Immunoglobulin 93
1.16.4 Prophylactic Aciclovir 93
1.16.5 Prophylactic Ganciclovir 94
1.16.6 Surveillance and Pre-emptive Therapy 95
1.17 New Approaches for the Prevention or Treatment of HCMV Infections in Allogeneic Stem Cell Transplant Recipients 96
1.17.1 Immunotherapy 96
1.17.2 HCMV vaccine 98
1.18 Aims of the Thesis 100

Chapter 2 Risk factors for HCMV Viraemia in Allogeneic Stem Cell Transplant Recipients in the Era of Pre-emptive Therapy 101
2.1 Introduction 101
2.2 Methods 103
2.2.1 Patients 103
2.2.2 Conditioning and GVHD Prophylaxis 104
2.2.3 Infection Prophylaxis 105
2.2.4 HCMV PCR Monitoring 106
2.2.5 HCMV Pre-emptive Therapy 106
2.2.6 Clinical Outcomes 106
2.2.7 Statistical Analysis 107
2.3 Results 107
2.3.1 Patients and Disease Characteristics 107
2.3.2 Transplant Related Outcomes 110
2.3.3 HCMV Infection 112
2.3.4 Analysis of the Risk Factors for HCMV Infection 115
2.3.5 Effect of In Vivo Campath Use on Time to HCMV Infection 118
2.3.6 Late HCMV Infection 124
2.3.7 HCMV Disease 129
2.4 Conclusions 130

Chapter 3 HCMV Replication Kinetics 135
3.1 Introduction 135
3.2 Methods 137
3.2.1 Patients 137
3.2.2 Extraction of DNA from Whole Blood 138
3.2.3 Real Time Quantitative PCR 139
3.2.4 Calculation of HCMV Replication Kinetics 142
3.3 Results 142
3.3.1 Patient outcomes 142
3.3.2 Quantitative HCMV PCR Results 147
3.3.3 HCMV Replication Kinetics 149
3.3.4 Predictors of Duration of Viraemia 149
3.3.5 Predictors of HCMV Disease 154
3.3.6 Viral Decay Curves and Response to Therapy 159
3.3.7 Comparison of Ganciclovir and Combination Ganciclovir/Foscarnet for the Treatment of HCMV Viraemia 164
3.3.8 Predictors of Recurrence of Infection 167
3.4 Conclusions 171

Chapter 4 HCMV Specific Immune Recovery following Allogeneic Stem Cell Transplantation 175
4.1 Introduction 175
4.2 Method 178
4.2.1 Patient Recruitment 178
4.2.2 Samples 178
4.2.3 Viral Load Monitoring 178
4.2.4 Conditioning Details and Infection Prophylaxis 179
4.2.5 Clinical Data 179
4.2.6 Mononuclear Cell Isolation 179
4.2.7 Antigen Stimulation 180
4.2.8 Intracellular Staining 181
4.2.9 FACS Analysis 182
4.2.10 Statistical Analysis 183
4.3 Results 183
4.3.1 Patients and Clinical Data 183
4.3.2 Results of FACS Analysis on Normal Controls 185
4.3.3 Immunophenotype of CD4+ T cells Responding to HCMV 190
4.3.4 Results of HCMV Specific Immune Function Monitoring in Allogeneic Stem Cell Transplant Recipients 194
4.3.5 Correlation of Immune Function with HCMV Infection 201
4.3.6 Effect of Campath-1H Use on HCMV Immune Recovery 210
4.3.7 Effect of Donor Serostatus on HCMV Immune Recovery 217
4.4 Conclusions 220

Chapter 5 Identification of HLA Class II Restricted Epitopes for the HCMV Proteins pp65 and IE1 223
5.1 Introduction 223
5.2 Methods 227
5.2.1 Patient Population 227
5.2.2 Antigen Stimulation and Intracellular Cytokine Staining 227
5.2.3 FACS Analysis 229
5.2.4 Epitope Prediction Modelling 229
5.2.5 HLA Typing 230
5.3 Results 230
5.3.1 HLA Typing 230
5.3.2 Results of HCMV Lysate and Peptide Pool Responses 232
5.3.3 Low Resolution Mapping 235
5.3.4 High Resolution Mapping 243
5.4 Conclusions 250

Chapter 6 General Discussion 250

Bibliography 267
Appendix A 327
Patient Consent for HCMV Immune Reconstitution Study 327
Patient Information Sheet 328
Appendix B 330
pp65 Peptide Pool Composition 330
IE1 Peptide Pool Composition 333

List of Tables

Table 1-1 Major non-infectious complications of allogeneic stem cell transplantation. 41
Table 1-2 Mechanisms by which HCMV evades host immune system 69
Table 2-1 Characteristics of transplant recipients. 109
Table 2-2 Univariable Cox Regression analysis of the impact of HCMV serology and infection on overall survival. 111
Table 2-3 Univariable Cox Regression analysis of the risk factors for HCMV infection 116
Table 2-4 Multivariable Cox Regression analysis of the risk factors for HCMV infection 117
Table 2-5 Univariable Cox regression analysis of the risk factors for late HCMV infection 125
Table 2-6 Multivariable Cox Regression analysis of the risk factors for late HCMV Infection. 126
Table 3-1 Clinical characteristics of 57 allogeneic stem cell transplant recipients experiencing HCMV infection between 1/1/01 and 31/12/04. 145
Table-3-2 Clinical characteristics of patients with HCMV viraemia and disease. 146
Table-3-3 Correlation of initial viral load, peak viral load, K_{max} and K_{mean} with total duration of HCMV viraemia in days 151
Table-3-4 Univariable logistic regression analyses of factors predicting a duration of viraemia of 14 days or more. 152
Table-3-5 Characteristics of immediate responders versus delayed responders to HCMV specific therapy. 161
Table-3-6 Multivariable logistic regression analysis of the risk factors for delayed response to therapy. 161
Table 3-7 Univariable Cox regression analysis for the risk factors for second HCMV infection. 168
Table 4-1 Characteristics and conditioning details of patients recruited for HCMV specific immune function monitoring following allogeneic stem cell transplant. 184
Table 4-2 Interferon γ response of normal controls to stimulation with HCMV lysate, pp65 peptide pool, and IE1 peptide pool. 187
Table 4-3 Immunophenotype of all CD4+ T cells and interferon gamma secreting CD4+ T cells in response to HCMV viral lysate, of patients and controls. 193
Table 4-4 Viral kinetics and HCMV specific immune response at time of infection of the allogeneic transplant patients experiencing HCMV infection 204
Table 4-5 Comparison of the median HCMV specific interferon gamma responses in allogeneic transplant recipients according to use of Campath-1H in vivo at different time points post transplant 212
Table 5-1 HLA Class II HCMV specific epitopes previously published for pp65 and IE1. 226
Table 5-2 HLA class I and II molecular typing results of normal controls. 231
Table 5-3 HLA class I and II molecular typing results of stem cell transplant patients. 231

Table 5-4 CD4+ T cell interferon gamma response of patients and controls to HCMV viral lysate, and the pp65 and IE1 peptide pools. 234
Table 5-5 HLA class II epitopes identified at high resolution. 246
Table 5-6 SYFPEITHI epitope prediction for HLA DRB1*0401 for the pp65 immunodominant sequence, HVLKAVFSRGDTPVLPHETRLLQ. 247
Table 5-7 SYFPEITHI epitope prediction for HLA DRB1*0301 for the pp65 immunodominant sequence, PPWQAGILARNLPMVATV. 247
Table 5-8 SYFPEITHI epitope prediction for HLA DRB1*1101 for the pp65 immunodominant sequence, PPWQAGILARNLVPMVATV 247

List of Figures

Figure 1-1 Electron micrograph image of the HCMV virion (Courtesy of Vincent Emery).....43
Figure 1-2 Map of the HCMV genome, reproduced with permission from Thomas Shenk (Murphy et al., 2003a).....48
Figure 1-3 Chest X-ray changes of HCMV pneumonitis in an allogeneic stem cell transplant recipient.....79
Figure 1-4 H&E stained slide of a colon biopsy of an allogeneic stem cell transplant patient with HCMV colitis showing typical viral inclusion bodies (personal collection).....83
Figure 1-5 Chemical structure of commonly used drugs for the treatment of human cytomegalovirus infections.....91
Figure 2-1 Kaplan Meier estimate of the cumulative incidence of HCMV infection in intermediate or high risk patients.....113
Figure 2-2 Kaplan Meier estimate of the cumulative incidence of HCMV infection according to HCMV serology.....114
Figure 2-3 Kaplan Meier estimate of the cumulative incidence of HCMV infection according to Campath in vivo use.....120
Figure 2-4 Kaplan Meier estimate of the cumulative incidence of HCMV infection in R+D+ patients according to Campath in vivo use.....121
Figure 2-5 Kaplan Meier estimate of the cumulative incidence of HCMV infection in R+D- patients according to Campath in vivo use.....122
Figure 2-6 Time to first HCMV infection according to Campath in vivo use...123
Figure 2-7 Kaplan Meier estimate of the cumulative incidence of late HCMV infection.....127
Figure 2-8 Kaplan Meier incidence of late HCMV infection according to HCMV serology.....128
Figure 3-1 Real Time PCR fluorescence emission plot for serially diluted control samples.....141
Figure 3-2 Standard curve for determination of HCMV viral load.....141
Figure 3-3 Frequency histogram of the initial HCMV viral load.....148
Figure 3-4 Frequency histogram of the peak HCMV viral load.....148
Figure 3-5 Duration of viraemia versus peak viral load.....153
Figure-3-6 Peak viral load according to the presence or absence of HCMV disease.....156
Figure 3-7 Duration of viraemia according to the presence or absence of HCMV disease.....157
Figure 3-8 Time to viral clearance following initiation of therapy according to the presence or absence of HCMV disease.....158
Figure 3-9 K_{max}, K_{mean}, and baseline viral load according to immediate or delayed response to HCMV therapy.....162
Figure 3-10 Rate of decline in HCMV viraemia according to the absence or presence of antiviral therapy.....163
Figure-3-11 HCMV decline rates before and after initiation of antiviral therapy.....163
Figure 3-12 Comparison of change in viral load at day seven between ganciclovir monotherapy and combination ganciclovir/foscarnet therapy.....165

Figure 3-13 Comparison of the change in viral load at four day intervals between ganciclovir monotherapy and combination therapy with ganciclovir/foscarnet. 166
Figure 3-14 Kaplan Meier estimate of the cumulative incidence of second HCMV infection. 169
Figure 3-15 Kaplan Meier estimate of the cumulative incidence of second HCMV infection according to donor HCMV serostatus. 170
Figure 4-1 Representative FACS plots of intracellular staining for interferon γ of CD4+ T cells following antigen stimulation. 188
Figure 4-2 HCMV viral lysate dose response curve. 189
Figure 4-3 Representative FACS plot of the immunophenotype of CD4+ T cells responding to HCMV viral lysate. 191
Figure 4-4 Comparison of the immunophenotype of the total CD4+ T cell population and CD4 T+ cells responding to HCMV viral lysate. 192
Figure 4-5 Representative plot of the HCMV specific immune response and viral load monitoring following transplant. 197
Figure 4-6 Scatter plots of the CD4+ T cell response to HCMV versus time post transplant. 198
Figure 4-7 Scatter plots of CD8+ T cell response to HCMV versus time post transplant. 199
Figure 4-8 The CD4+ and CD8+ T cell interferon gamma response to HCMV post transplant. 200
Figure 4-9 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV 205
Figure 4-10 Kaplan Meier estimate of the cumulative incidence of CD8+ T cell immune recovery to HCMV 206
Figure 4-11 CD4+ T cell response to HCMV viral lysate according to the presence or absence of HCMV infection. 207
Figure 4-12 CD4+ T cell response to pp65 and IE1 peptide pools according to the presence or absence of HCMV infection. 208
Figure 4-13 CD8+ T cell response to pp65 and IE1 peptide pools according to the presence or absence of HCMV infection. 209
Figure 4-14 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV according to Campath-1H use 213
Figure 4-15 Kaplan Meier estimate of the cumulative incidence of CD8+ T cell immune recovery to HCMV 214
Figure 4-16 CD4+ T response to HCMV according to use of Campath-1H in vivo 215
Figure 4-17 CD8+ T Response to HCMV according to use of Campath in vivo. 216
Figure 4-18 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV according to the HCMV serology of the donor and recipient. 218
Figure 4-19 Kaplan Meier plot of the cumulative incidence of CD8+ T cell immune recovery to HCMV according to the HCMV serology of the donor and recipient. 219
Figure 5-1 Representative FACS plots of the CD4+ T Cell interferon gamma response to pp65 low resolution mapping pools. 237
Figure 5-2 Representative FACS plots of the CD4+ T Cell interferon gamma response to IE1 low resolution mapping pools. 238

Figure 5-3 Results of low resolution pp65 mapping of normal controls and patients. 239
Figure 5-4 Results of low resolution IE1 mapping of controls and patients..... 240
Figure 5-5 Percentage of patients and controls responding to each low resolution pp65 mapping pool..... 241
Figure 5-6 Percentage of patients and controls responding to each low resolution IE1 mapping pool. 241
Figure 5-7 Comparison of the number of IE1 and pp65 peptide pool responses between patients and controls. 242
Figure 5-8 Representative FACS plots of high resolution peptide mapping for the identification of an immunogenic epitope from pp65 peptide pool 1..... 248
Figure 5-9 Interferon gamma response as a percentage of total CD4+ T Cells following stimulation with individual peptides from pp65 peptide pool 1. 249
Figure 5-10 Predicted anchoring residues of the pp65 epitope, LLQTGIHVRVSQPSL for DQA1*0103/DQB1*0602. 249

Abbreviations

ADCC = Antibody dependent cell-mediated cytotoxicity
AML = Acute myeloid leukaemia
APC = Allophycocyanin
APC = Antigen presenting cell
ATG = Anti-thymocyte globulin
BAL = Bronchoalveolar lavage
BEAM = Carmustine, etoposide, cytosine arabinoside and melphalan
BSA = Bovine serum albumin
CLL = Chronic lymphocytic leukaemia
CML = chronic myeloid leukaemia
CPE = Cytopathic effect
CTL = Cytotoxic T cell
D = Donor
DEAFF = Detection of early antigen fluorescent foci
DFS = Disease free survival
DLI = Donor lymphocyte infusion
DMSO = dimethyl sulphoxide
EBMT = European Group for Blood and Marrow Transplantation
EBV = Epstein Barr Virus
FAM = 6-carboxy-fluorescein (fluorescent dye used in Taqman)
FCS = Fetal calf serum
FITC = Fluorescein isothiocyanate
G-CSF = Granulocyte colony stimulating factor
GM-CSF = Granulocyte macrophage colony stimulating factor
GVHD = Graft versus host disease
H&E = Haematoxylin and Eosin
HCMV = Human Cytomegalovirus
HEPA = High efficiency particulate air filter
HIV = Human immunodeficiency virus
HLA = Human leukocyte antigens
HSV = Herpes simplex virus
IBMTR = International bone marrow transplant registry
IE1 = Immediate early protein 1
IFγ = Interferon gamma
IL2 = Interleukin 2
IL10 = Interleukin 10
IM = Idiopathic myelofibrosis
Kdecline = Mean rate of viral decline (day-1)
Kmax = Maximum rate of viral rise (day-1)
Kmean = Mean rate of viral rise (day-1)
LIR1 = Leukocyte immunoglobulin like receptor 1
MCP = Monocyte chemotactic protein 1
MDS = myelodysplasia
MHC = Major histocompatibility complex
MIE = Major immediate early
NFkappaB = Nuclear Factor Kappa B

NK = Natural killer
NMDP = National marrow donor program
ORF = Open Reading Frame
PBMC = Peripheral blood mononuclear cells
PBSC = Peripheral blood stem cells
PCR = Polymerase Chain Reaction
PE = Phycoerthyrin
PerCP = Peridinin chlorophyll protein
PMA = Phorbol 12-myristate 13-acetate
PMN = Polymorphonuclear leukocytes
pp65 = Phosphoprotein 65
PTLD = Post Transplant Lymphoproliferative Disorder
R = Recipient
RH = Relative hazard
RIC = Reduced intensity conditioning
RT-PCR = Reverse transcriptase polymerase chain reaction
SEB = Staphyloccocal endotoxin B
TA = Thoraco-abdominal (irradiation)
TAMRA = 6-carboy-tetramethyl-rhodamine
TAP = Transporter of antigen processing
TBI = Total body irradiation
TCD = T-cell depletion
TCM = Central memory
TEM = Effector memory
TLI = Total lymphoid irradiation
TNFα = Tumour necrosis factor alpha
TREC = T-cell receptor rearrangement excision circles
TRM = Treatment related mortality
VZV = Varicella Zoster Virus

Definitions and Conventions

The following terms with reference to HCMV are defined for the purpose of the thesis

Infection = the detection of HCMV by PCR, and may occur in the presence or absence of HCMV disease.

Primary infection = infection in a patient not previously infected with HCMV.

Reactivation = the detection of HCMV viraemia by PCR in a patient with proven previous HCMV infection as evidenced by HCMV positive serology.

Latency = "the persistence of viral genome in the absence of production of infectious virions, but with the ability of the viral genome to reactivate after specific stimuli" (Sinclair and Sissons, 2006).

HCMV Disease = the presence of end organ damage due to HCMV infection.

HCMV Serostatus = The serostatus of recipient and donor pairs are expressed with recipient serostatus first and donor serostatus second. It is acknowledged that this differs from the convention used in solid organ transplantation but has been used due to the greater importance of the recipients serostatus on the risk of HCMV infection or reactivation in allogeneic stem cell transplantation. A + sign is used to indicate a positive serology and a – sign is used to indicate a negative serology. Thus:

R+D+ = Recipient HCMV seropositive/Donor HCMV seropositive
R+D- = Recipient HCMV seropositive/Donor HCMV seronegative
R-D+ = Recipienet HCMV seronegative/Donor HCMV seropositive
R-D- = Recipient HCMV seronegative/Donor HCMV seronegative

Acknowledgements

This thesis would not have been possible without the support of the following people:

Professor Vince Emery, my supervisor. I thank him for his enthusiastic supervision and encouragement, for providing me with the opportunity to undertake this work, for supervising my research, and providing me with constructive comments on my thesis.
Professor Paul Griffiths, I am grateful for supporting and encouraging me throughout my research, and for all his positive feedback.
I also thank all my other friends and colleagues at the Centre for Virology for their help and advice, including Dr Aycan Walker, Kerissa Lawson, Gill Clewley and Dr Kuba Koycinski.

From the Centre for Virology, Department of Infection, Royal Free and University College Medical School, London.

Professor H Grant Prentice, for providing me with the opportunity and inspiration to do this work.
Professor Stephen Mackinnon and Dr Mike Potter, for the support and encouragement in the clinical aspects of this thesis.
Dr Mark Lowdell, for teaching me the principles and practice of flow cytometry and for his inspiration and support.
I also thank my colleagues in the Department of Haematology. In particular, I am very grateful to Janet North for her kind patience in teaching me the skills needed for the immune stimulation experiments used in Chapter Four and Five.

Department of Haematology, Royal Free and University College Medical School, London

Professor Alejandro Madrigal. For his support and encouragement
Professor Toni Dodi. For his support and advice in the design of the antigen specific immunology experiments.
Dr Samantha Paston for her help and encouragement.
I also thank Hazel Forde for teaching me many of the laboratory skills I needed to complete this work.

Anthony Nolan Reseach Institute, London

Professor Caroline Sabin. For her advice and critical feedback on the statistical analysis of this thesis.

Department of Primary Care & Population Sciences, Royal Free and University College Medical School, London

On a more personal note....

I thank my wife and companion, Dr Catherine Rachel Lawson, to whom I am indebted for her kind and loving support, for her encouragement, for proof reading the thesis, and for putting up with being neglected for long periods of time during this course of study. I will make it up to you.

I also wish to thank all my family and friends for their constant encouragement and enthusiasm

Finally, to my children, Benjamin, Alexander, and Zachary, I thank for providing me with the motivation to complete this work so I can spend more time with them.

Introduction

1.1 Allogeneic Stem Cell Transplantation

1.1.1 A Historical Perspective

Although the first recorded organ transplant was performed in 1906 by Alexis Carrel, a British surgeon and pioneer in vascular surgery who described the xenotransplant of a human kidney (Jaboulay, 1906), the ground work for transplantation was laid by the Australian virologist, Frank Burnet, a Noble laureate, with his hypothesis of the immunological recognition of self. Medawar, who was also awarded the 1960 Noble Prize for Medicine or Physiology, first described acquired immune tolerance, showing that the infusion of allogeneic bone marrow before immune competence in mice would result in tolerance of skin grafts from the bone marrow donor but not from a third animal (Billingham et al., 1953). However, solid organ transplantation in humans did not become routine until the pioneering work of Hume and colleagues at Harvard Medical School, Boston, who elegantly described the clinical and pathological features of graft rejection in a series of 9 renal transplants (Hume et al., 1955). Murray, who was awarded the Nobel Prize for Medicine in 1990, reported the first successful human allograft of a kidney in an identical twin (Guild et al., 1955). Renal transplantation has now become commonplace clinical practice. However, the field of bone marrow transplantation proved to be more problematic.

1.1.2 Early Scientific Experiments

Early attempts at infusion of human bone marrow to treat aplastic anaemia were unsuccessful (Osgood et al., 1939). However, the development of the atomic bomb and its devastating effects in the Second World War stimulated research into radiation and its effect on the bone marrow. Jacobson laid the groundwork for the concept of bone marrow transplantation when he demonstrated that shielding the spleen protected mice from otherwise lethal doses of ionizing radiation (Johnson et al., 1949). Lorenz then showed that intra peritoneal infusion of bone marrow from a second mouse resulted in recovery of bone marrow function in lethally irradiated mice (Lorenz et al., 1951). It was subsequently shown that when irradiated mice are infused with allogeneic bone marrow, they no longer reject skin grafts from the donor animal (Main and Prehn, 1955). As immunosuppressive drugs were not available at this time, one of the main stimuli to develop marrow transplantation came from the desire to allow a recipient to tolerate kidney allografts.

1.1.3 Bone Marrow Transplant Pioneers

In 1957, E. Donnell Thomas, arguably the father of bone marrow transplantation and later a Noble laureate, using large quantities of marrow reported transient engraftment in one patient (Thomas et al., 1957). Two years later Thomas reported successfully transplanting two leukaemia patients with bone marrow from their identical twins (Thomas et al., 1959), although both patients relapsed a few months later. Mathé, another early pioneer in the field of marrow transplantation,

unsuccessfully transplanted six victims of a radiation accident in Yugoslavia in 1959 (Mathe et al., 1959). He later reported the first bone marrow transplant in a patient with acute leukaemia to survive longer then one year, although the patient died after 20 months from infection as a complication of chronic graft versus host disease (Mathe et al., 1963). Further clinical experience with bone marrow transplants in the 1950's and 60's were largely unsuccessful, thwarted either by failure of engraftment or overwhelming graft versus host disease (GVHD). During this time, extensive experiments were being undertaken on dogs at the Fred Hutchinson Cancer Centre in Seattle under the direction of Thomas. It was shown that following supra-lethal doses of radiotherapy, dogs could recover if infused with autologous marrow harvested prior to irradiation (Mannick et al., 1960). Furthermore, the harvested marrow could be frozen in dimethylsulfoxide (DMSO) and successfully re-infused into an irradiated dog at a later date (Thomas and Ferrebee, 1962; Cavins et al., 1962). However, allogeneic transplantation in dogs remained largely unsuccessful due to either GVHD or failure of engraftment, with the exception of the occasional transplant between litter-mates (Thomas and Ferrebee, 1962).

1.1.4 Tissue Typing and Graft Versus Host Disease

According to Billingham, there are three requirements for the development of GVHD: the graft must contain immunologically competent cells, the recipient must be unable to mount an effective immune response and the recipient must express antigens not present in the donor (Billingham, 1966). Acute GVHD occurs in the first hundred days

following transplant and is characterised by dermatitis, enteritis, and hepatitis. It is graded according to the criteria published by Glucksberg (Glucksberg et al., 1974). Chronic GVHD by definition occurs after one hundred days, either as continuation of acute GVHD or as a de novo presentation. Historically, chronic GVHD was graded as limited or extensive according to criteria described by Shulman (Shulman et al., 1980), although a new grading system based on the extent of skin involvement (greater than 50% of body surface area), presence of thrombocytopeania and a progressive-type onset has been proposed (Akpek, 2002).

In the early 1960's, as a result of the pioneering work of Dausset (Dausset, 1958), van Rood (van Rood and van Leeuwen, 1963), Payne and Bodmer (Payne et al., 1964), polymorphisms within the human lymphocyte antigen (HLA) complex were identified, allowing histocompatibility testing to be performed between potential bone marrow donors and recipients. Using histocompatibility testing, the first HLA matched sibling bone marrow transplant was performed for a child with immunodeficiency (Gatti et al., 1968). In the late 1970's Thomas reported his experience of 100 bone marrow transplants, performed for patients with acute leukaemia, from HLA identical siblings, conditioned with cyclophosphamide 120mg/kg over two days and total body irradiation (TBI) 10 Gy, with only one graft rejection (Thomas et al., 1977). However, despite matching by HLA, half the patients developed moderate to severe GVHD, many requiring treatment with anti-thymocyte globulin (ATG). In addition, interstitial pneumonitis developed in 54 patients with a fatal

outcome in 34 patients, and the most common etiologic agent was human cytomegalovirus (HCMV), often in association with GVHD. This is one of the first studies to document the severity of the impact of HCMV infection on stem cell transplantation. An important observation at this time was that the presence of GVHD reduced the likelihood of leukaemic relapse, the first description of graft-versus-leukaemia effect (Weiden et al., 1979).

1.1.5 Post Transplant Immunosuppression and Refinements of the Conditioning Regimen

Methotrexate administered post bone marrow transplantation had previously been shown to ameliorate GVHD in dogs (Storb et al., 1970). Based on this knowledge, the Seattle team introduced a regimen of methotrexate 10mg/m^2 on days 1, 3, 6 and 11 following infusion of the donor bone marrow (Storb et al., 1977; Storb et al., 1983; Thomas et al., 1975a; Thomas et al., 1975b), later also incorporating ciclosporin in combination with methotrexate. More recently, the combination of ciclosporin and mycophenolate has been evaluated for the prevention of graft versus host disease following stem cell transplantation (Bornhauser et al., 1999).

Another important early development in reducing the long term toxicity of bone marrow transplantation has been fractionation of TBI (Deeg et al., 1986; Thomas et al., 1982), later incorporated into a protocol of cyclophosphamide 60mg/kg for 2 days and 12 Gy fractionated TBI. At the same time, Santos at the John Hopkins Medical School developed a non-TBI regimen using busulfan 16mg/kg over 4 days and cyclophosphamide 120mg/kg over two days (Santos et al., 1983).

1.1.6 T-cell Depletion

Another approach to reducing the risk of GVHD has been to deplete the donor graft of T-cells, referred to as T-cell depletion (TCD). Early attempts to deplete the graft of T-cells involved the in-vitro addition of murine anti-thymocyte globulin (ATG) and rabbit complement to the graft (Martin et al., 1985). Although this significantly decreased the incidence of acute GVHD, graft failure occurred in eight out of the 20 HLA-matched patients transplanted in this way, and to overcome this it was necessary to increase the intensity of the conditioning regimen. A further downside of T-cell depletion is that it results in a significantly increased risk of leukaemia relapse (Goldman et al., 1988). The use of monoclonal antibodies for T-cell depletion was pioneered by Prentice and colleagues in 1982 (Prentice et al., 1982), who showed that the incidence of acute GVHD was reduced from 79% (based on an earlier cohort of 14 patients) to 18% in 17 patients T-cell depleted with the murine monoclonal antibody to CD3, OKT3. It should be noted that in this cohort two patients died of disseminated HCMV infection, another recognized complication of T-cell depletion. Using a cocktail of two potent monoclonal T-cell antibodies, resulting in the removal of greater than 99% donor lymphocytes from the graft, the same team reported successful engraftment in 13 patients with no acute GVHD score greater than 1 despite the absence of post-transplant immunosuppression (Prentice et al., 1984). In 1984, Waldmann introduced the rat derived anti-CD52 monoclonal antibody, Campath-1 to the transplant community, when he described its use for T-cell depletion in 11 HLA-matched transplant

patients (Waldmann et al., 1984), although two of the patients experienced late graft failure. Physical methods to deplete the graft of lymphocytes while retaining sufficient stem cells for engraftment have also been employed, such as counter flow centrifugation (Wagner et al., 1988; de Witte et al., 1986) and immuno-absorption columns (Dreger et al., 1995). Despite improvements in T-cell depletion, graft failure and increased relapse rates continue to be a significant problem, with the risk of graft rejection for HLA-matched sibling transplants with T-cell depleted grafts being 9 times that for non T-cell depleted grafts (Marmont et al., 1991).

1.1.7 Unrelated Stem Cell Transplants

A major limiting factor in bone marrow transplantation has been the availability of suitable donors. The ABO blood group barrier was overcome by Graw in 1974, when he transplanted a blood group A donor into a blood group O recipient, using plasmapheresis and Witensky's A substance to reduce the anti-A titre (Graw, Jr. et al., 1974), although grafts from donors with major ABO mismatch are now red cell depleted using automated cell separators. However, for potential stem cell transplant recipients, a sibling has only a 25 percent chance of being HLA-identical. As the average family size in the western world continues to fall, this limits the number of patients for whom sibling donors are available. Tissue typing of extended family members may identify an HLA identical non-sibling related donor in 10 percent of patients, with comparable results to HLA-matched sibling donor transplants (Beatty et al., 1985), but this still leaves 70 percent of patients without a matched

related donor. Using serological matching at the HLA-A, B and DR1 loci to identify matched unrelated donors (so called 6 out of 6 match), successful outcomes following bone marrow transplant have been reported (Hansen et al., 1998), although the incidence of graft failure and severe GVHD is greater than HLA-identical siblings (Kernan et al., 1993; McGlave et al., 1990) . Further improvements in transplant results have been obtained using molecular HLA typing techniques for both Class I A, B and C loci (Sasazuki et al., 1998), and class II DRB1 loci (Devergie et al., 1997; Petersdorf et al., 1995). In order to facilitate the availability of matched unrelated bone marrow donors, large national registries have been established, the first being the Anthony Nolan Trust in the United Kingdom, with over 360,000 donors (www.anthonynolan.org.uk). The largest registry internationally is the National Marrow Donor Program (NMDP) in the United States with over six million donors (www.nmdpresearch.org), and through the International Bone Marrow Transplant Registry (IBMTR), patients have access to over 10 million potential donors. For Caucasian patients, using sophisticated search algorithms the chance of finding at least a six out of six HLA matched unrelated donor through the IBMTR is 83 percent although for other ethnic groups this is much lower.

1.1.8 Alternative Donor Sources

For patients without a matched related or unrelated donor, the Perugia group pioneered the technique of haplo-identical transplants (Aversa et al., 2005; Aversa et al., 1998). Intensive conditioning regimens and T-cell depletion by CD34 selection were used to overcome the problem of graft

rejection and severe graft versus host disease. The Japanese on the other hand have successfully used the concept of feto-maternal chimerism (Burlingham et al., 1998) to perform non T-cell depleted haplo-identical transplants (Ichinohe et al., 2004; Narimatsu et al., 2004; Obama et al., 2004; Yoshihara et al., 2004). Another alternative to unrelated donor marrows is the use of umbilical cord blood transplants, which allow a greater degree of HLA mismatch with reduced rates of GVHD (Schoemans et al., 2006). Although umbilical cord blood transplants are associated with high rates of graft failure, this can be overcome by the use of double umbilical cord transplants (Barker et al., 2005; Majhail et al., 2006).

1.1.9 Peripheral Blood Stem Cell Transplants

Sufficient peripheral blood stem cells (PBSC) to permit bone marrow recovery following myeloablative chemotherapy can be harvested by apheresis from patients with malignant disease after stimulation with granulocyte colony stimulating factor (G-CSF) (Bensinger et al., 1993). This prompted the harvesting of G-CSF stimulated PBSCs from healthy donors for the purpose of allogeneic transplantation (Dreger et al., 1993; Russell et al., 1993). Using PBSCs for allogeneic stem cell transplants results in earlier platelet and granulocyte engraftment, but is associated with a higher risk of acute (Schmitz et al., 2002) and chronic GVHD (Blaise et al., 2000) in some but not all studies (Bensinger et al., 2001; Powles et al., 2000). The CD34 cell dose of the graft influences outcome, as the use of peripheral blood stem cells with a high CD34 dose (greater then $8.3X10^6$/Kg) for HLA identical sibling transplants have been shown

to increase mortality due to chronic GVHD when compared to low dose grafts (Mohty et al., 2003a). Both cell dose and stem cell source influence outcome. In HLA identical sibling stem cell transplants for standard risk leukaemia, the best outcome is for patients receiving bone marrow with a high (greater then $2.7X10^8$) cell dose (Gorin et al., 2003), although in high risk leukaemia patients, the best outcome is seen with the use of PBSC (Bensinger et al., 2001).

1.1.10 Reduced Intensity Conditioning Transplants

Due to the toxicity associated with fully myeloablative conditioning regimens, advanced age and significant comorbidity have excluded many patients from being candidates for allogeneic stem cell transplantation. In order to reduce toxicity and offer allogeneic transplants to a greater number of patients, so called non-myeloablative or reduced intensity conditioning (RIC) transplant regimens have been developed. The first RIC conditioning regimen was in fact used in Seattle for the treatment of aplastic anaemia, consisting of cyclophosphamide 50mg/kg for four days (Storb et al., 1974). Modern reduced intensity conditioning regimens use one of three approaches; low dose TBI with or without fludarabine (McSweeney et al., 2001), fludarabine and an alkylating agent (Giralt et al., 1997), or T-cell antibodies such as ATG (Slavin et al., 1998) or Campath-1H (alemtuzumab) (Kottaridis et al., 2000) in combination with a purine analogue and alkylating agent. One third of allogeneic stem cell transplants now incorporate a reduced intensity conditioning regimen (Gratwohl et al., 2006a).

1.1.11 Donor Lymphocyte Infusion

Donor lymphocyte infusions (DLI) were first successfully employed to augment the donor derived anti-leukaemia effect for patients relapsing following allogeneic transplantation for chronic myeloid leukaemia (CML). DLIs have also been used to treat relapsed acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) and myelofibrosis following myeloablative allogeneic transplants (Kolb et al., 1990), although this carries a high risk of also inducing graft-versus host disease. In the setting of CML, the best results are obtained if DLI is performed at the time of molecular or cytogenetic relapse and before full haematological relapse (van Rhee et al., 1998). Mixed donor chimerism is predictive of an increased risk of relapse of CML (Mackinnon et al., 1994), and acute leukaemia (Bader et al., 2000; Mattsson et al., 2001). With the advent of reduced intensity conditioning regimens, DLIs have also been used to convert patients to full donor chimerism, although with variable results (Bethge et al., 2004; Marks et al., 2002). A graft-versus tumour effect following DLI in patients transplanted with a reduced intensity conditioning regimen for Hodgkin's disease, Non-Hodgkin's Lymphoma and multiple myeloma has been demonstrated (Peggs et al., 2003a; Peggs et al., 2005; Morris et al., 2004).

1.2 Indications for Allogeneic Transplantation

Since the late 1970s, the number of allogeneic transplant procedures has been increasing steadily. In 2004 the number of first allogeneic transplant procedures reported to the European Group for Blood and Bone Marrow

Transplantation (EBMT) was 7,407 (Gratwohl et al., 2006a), with the majority performed for lymphoid and myeloid malignancies. With continued improvements in supportive care and new technologies, the indications for allogeneic stem cell transplantation continue to widen.

1.2.1 Acute Myeloid Leukaemia and Myelodysplasia

Notwithstanding improvements in chemotherapy the majority of patients with acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) succumb to resistant or relapsed disease (Burnett et al., 2002). In patients with poor or intermediate risk AML based on cytogenetic profiles, allogeneic stem cell transplant when used as first line therapy results in significantly better disease free survival (DFS) in a donor versus no donor analysis in three large prospective collaborative studies (Burnett et al., 2002; Suciu et al., 2003; Cornelissen et al., 2007). A meta analysis of all three studies demonstrates a 12 percent overall survival benefit by donor availability for patients without a favourable cytogenetic profile (Cornelissen et al., 2007). In good risk patients, such as those with t(8:21), inv(16) and t(15:17), the treatment related mortality (TRM) of transplantation outweighs the benefit. Transplantation offers the best chance of cure in patients with relapsed AML (Breems et al., 2005). Patients with primary refractory disease generally have a dismal outlook, although Kolb's group have reported promising results using sequential therapy with fludarabine, high dose cytarabine and amsacrine chemotherapy followed three days later by a RIC transplant (Schmid et al., 2005). The role of RIC transplant for AML patients unsuitable for conventional intensity conditioned transplants has also been evaluated,

and a recent EBMT working party retrospective analysis comparing autografting with RIC transplants in patients over 50 years of age showed for patients in first complete remission, RIC is associated with a better DFS at the cost of a greater TRM. For patients in second complete remission, the TRM was lower for RIC with a DFS at one year of 76 percent compared to 31 percent for autografts (Herr et al., 2007).

1.2.2 Acute Lymphoblastic Leukaemia

Prior to the adaptation of combination therapy used for paediatric ALL, adult ALL was invariably fatal. However, only about 30 percent of adults are long term disease-free survivors following intensive chemotherapy and this is even lower for relapsed ALL (Pui and Evans, 2006). A French study comparing chemotherapy, autologous transplantation and allogeneic transplantation for patients in first complete remission found that allogeneic stem cell transplantation showed a superior outcome for high risk but not standard risk ALL patients (Thiebaut et al., 2000). The Japanese found no difference in 6 year survival between patients allocated an HLA identical sibling allogeneic transplant or chemotherapy, although Ph+ ALL patients did show superior survival with allogeneic transplantation (Takeuchi et al., 2002). The EORTC ALL-3 trial comparing an autologous transplant and maintenance chemotherapy with an allogeneic transplant in patients in first complete remission under 50 years of age also failed to show a survival advantage for allogeneic transplantation (Labar et al., 2004). A current evidence based review of the published literature supports the view that allogeneic stem cell transplantation in first complete remission should be reserved for high risk

ALL patients (Hahn et al., 2006). Age is one of the most important prognostic risk factors for ALL but advancing age is also associated with an increasing TRM. In an attempt to reduce the TRM, RIC transplants have been evaluated for ALL but are associated with a high relapse risk (Hamaki et al., 2005).

1.2.3 Chronic Myeloid Leukaemia

Since the first description of allogeneic stem cell transplantation for chronic myeloid leukaemia (Fefer et al., 1979), allogeneic stem cell transplantation has been recognized as the only therapy with curative potential in chronic myeloid leukaemia. Long term disease free survival rates vary between 30 to 80 percent, depending on the stage of disease at transplantation (Clift et al., 1993; Craddock et al., 2000; Goldman et al., 1988; Thomas et al., 1986). A graft versus leukaemia effect is well described (Weiden et al., 1979), and the risk of relapse is increased with T cell depletion (Goldman et al., 1988). This graft versus leukaemia effect can be utilised to treat relapse following allogeneic stem cell transplantation by the use of DLIs (Barrett et al., 1998; Collins, Jr. et al., 1997; Dazzi et al., 2000; Guglielmi et al., 2002; Kolb et al., 1990; Porter et al., 2000). RIC regimens are increasingly being employed because of the reduced transplant related mortality (Crawley et al., 2005). However, since the introduction of targeted molecular therapy for CML with the BCR:ABL tyrosine kinase inhibitor imatinib, the number of allogeneic transplant procedures performed for CML has fallen (Gratwohl et al., 2006b), as most patients are now offered imatinib and proceed to transplant only if imatinib fails.

1.2.4 Aplastic Anaemia

The most commonly employed conditioning regimen for aplastic anaemia is cyclophosphamide 200 mg/kg and ATG 30mg/kg for three days with methotrexate and ciclosporin for GVHD prophylaxis, and was pioneered in Seattle (Storb et al., 1994). The outcome for HLA identical sibling transplants is good, with long term survival rates of greater than 80 percent (Bacigalupo et al., 2000; Kahl et al., 2005), although graft rejection remains a significant problem in between 5-15 percent of patients. This compares favourably with an overall survival of 70 percent at 3-4 years for immunosuppressive therapy with ATG and ciclosporin (Frickhofen et al., 2003). Recipients of matched unrelated bone marrow transplants for severe aplastic anaemia fare significantly worse with higher rates of acute and chronic GHVD, and a five year overall survival rate of between 39 and 61 percent (Bacigalupo et al., 2005; Deeg et al., 2006; Kojima et al., 2001; Passweg et al., 2006). The role of allogeneic stem cell transplantation in the inherited bone marrow failure syndromes such as Fanconi anaemia is also well established (Dokal, 2003).

1.2.5 Thalassaemia and Sickle Cell Anaemia

Thalassaemia major results in lifelong transfusion dependence with the incumbent consequences of iron overload and shortened life expectancy. Lucarelli pioneered stem cell transplantation as a curative procedure for thalassaemia (Lucarelli et al., 1984), and showed that if patients were transplanted before they developed hepatomegaly, portal fibrosis or significant iron overload, excellent results could be achieved (Lucarelli et

al., 1990). The first allogeneic transplant in a patient with sickle cell disease was reported in 1984 for a patient also suffering from acute myeloid leukaemia (Johnson et al., 1984). The best results have been obtained in paediatric sickle cell patients with HLA identical sibling transplants (Vermylen et al., 1998; Walters et al., 2000). Umbilical cord blood transplantation has also been evaluated for both thalassaemia and sickle cell disease (Locatelli et al., 2003).

1.2.6 Other Haematological Conditions

The treatment of choice for relapsed high grade lymphoma after salvage chemotherapy is high dose chemotherapy followed by an autologous transplant (Philip et al., 1987). Limited treatment options are available to patients who relapse following autologous transplantation. A graft versus lymphoma effect has been demonstrated following myeloablative allogeneic stem cell transplantation (Jones et al., 1991). However the TRM following allogeneic stem cell transplantation for lymphoma is high at up to 40 percent, and following a failed autografting rises to between 50 and 80 percent (Chopra et al., 1992; de Lima M. et al., 1997; Jones et al., 1991; Tsai et al., 1997; Verdonck et al., 1997). Reduced intensity conditioning regimens have generally failed to show any improvement in outcomes for high grade lymphomas (Morris et al., 2004; Robinson et al., 2002). Low grade lymphoma which is incurable by conventional chemotherapy and autologous transplantation, has shown promising results following reduced intensity conditioning transplantation (Escalon et al., 2004; Khouri et al., 2001; Robinson et al., 2002), especially when combined with DLI (Faulkner et al., 2004; Morris et al., 2004).

Transplantation of relapsed Hodgkin's disease using a RIC regimen has also shown encouraging results (Peggs et al., 2005).

Chronic Lymphocytic Leukaemia (CLL) is a disease with a variable prognosis, but fludarabine refractory disease has a very poor prognosis with a median survival of eight months (Keating et al., 2002). Although allogeneic transplantation can result in lasting remission (Khouri et al., 1997; Khouri et al., 2002), mortality with conventional conditioning regimens is high with a TRM of between 31 and 60 percent (Doney et al., 2002; Michallet et al., 1996; Pavletic et al., 2000). Reduced intensity conditioning regimens have also been used for CLL (Delgado et al., 2006; Khouri et al., 2004; Schetelig et al., 2003), and a retrospective EBMT study has demonstrated a reduced TRM (Dreger et al., 2005).

Idiopathic myelofibrosis (IM) has also been successfully treated with allogeneic stem cell transplantation, with overall survival rates of between 39 and 77 percent when conventional intensity conditioning regimens are used (Daly et al., 2003; Deeg et al., 2003; Anderson et al., 1997). Recent reports using RIC regimens for IM have shown extremely good overall survival rates of between 84 and 100 percent (Devine et al., 2002; Kroger et al., 2005; Merup et al., 2006; Rondelli et al., 2005).

1.2.7 Congenital Immunodeficiency Syndromes and Other Non-Malignant Conditions

Since the first report of a successful bone marrow transplant for severe combined immunodeficiency (Gatti et al., 1968), stem cell transplantation is now performed for a wide range of immune deficiency syndromes including severe combined immunodeficiency, Wiskott Aldrich syndrome,

chronic granulomatous disease, and leukocyte adhesion deficiency. Allogeneic transplants are also performed to treat inherited metabolic disorders such as Hurlers syndrome, adrenoleukodystrophy and osteopetrosis although they are generally most effective before the patient is symptomatic (Yeager, 2002).

1.3 Complications of Transplantation

Since the development of allogeneic stem cell transplantation, treatment related mortality has steadily improved (Gratwohl et al., 2006b). This has largely been because of improvements in supportive care, such as better control of GVHD and the prevention and treatment of infectious complications.

1.3.1 Infectious Complications

Due to the immunosuppressed nature of allogeneic stem cell transplant recipients, patients are at a greatly increased risk of opportunistic bacterial, fungal, and viral infections. Deaths from infections account for 11% of all deaths following transplantation for early leukaemia, with 36% due to bacteria, 31% due to viruses and 28% due to fungi (Gratwohl et al., 2005). The use of empiric antibiotics for the management of neutropaenic fever has reduced the death rate from bacterial infections, but the emergence of bacterial resistance such as vancomycin-resistant enterococcus represents an increasing hazard (Avery et al., 2005). While Candida albicans is effectively prevented by the use of prophylactic azoles, invasive aspergillus infections remain a major cause of mortality

following allogeneic transplant, although newer agents are becoming available (Marr et al., 2002).

The most common viral infection complicating stem cell transplantation is HCMV and is discussed in further detail in the following sections. Reactivations of other latent herpes viruses are also responsible for significant morbidity and mortality. Prophylaxis with aciclovir is highly effective at preventing reactivation of Herpes simplex (HSV) infections. Ebstein Barr Virus (EBV) reactivation can result in EBV associated Post Transplant Lymphoproliferative Disease (EBV-PTLD). Established EBV-PTLD carries a high mortality even when treated by DLI and the monoclonal anti-CD20 antibody, Rituximab (Loren et al., 2003). Risk factors for EBV reactivation include T-cell depletion, reduced intensity conditioning and recently umbilical cord transplants (Brunstein et al., 2006). Polymerase chain reaction (PCR) monitoring to guide pre-emptive therapy either by modulation of immunosuppression or use of Rituximab has been shown to be effective in preventing the development of EBV-PTLD (Cesaro et al., 2005; Kinch et al., 2007). Immunotherapy with donor derived EBV specific cytotoxic T cells has also been shown to be effective (Liu et al., 2002). Reactivation of Human Herpes Virus 6 (HHV6) has been associated with delayed platelet engraftment and can cause encephalitis and hepatitis (Ljungman et al., 2000). Reactivation of varicella zoster virus (VZV) tends to occur late after transplantation, and usually presents as a limited herpes zoster (shingles) infection but may cause a disseminated varicella infection.

Among the non-herpes viruses complicating allogeneic stem cell transplantation, one of the most important is adenovirus, reported to occur in between 5 to 21 percent of patients following stem cell transplantation, with a mortality of up to 50 percent (Chakrabarti et al., 2002b). Paediatric transplant patients are at a particularly high risk, and viral monitoring of blood and stool, with pre-emptive cidofovir is recommended, while adoptive immunotherapy is under evaluation (Feuchtinger et al., 2006). Respiratory viruses are also important pathogens in allogeneic stem cell transplant recipients. A prospective multicentre EBMT study documented an incidence of lower respiratory tract infection due to respiratory viruses of 2.1 percent, with respiratory syncytial virus and influenzae A being the most common (Ljungman et al., 2001b). BK virus is associated with painful hemorrhagic cystitis, and related donors are at greatest risk, while RIC transplants appear to be protective (Erard et al., 2005; Giraud et al., 2006).

The hepatitis viruses, particularly hepatitis B can also be a major problem following transplantation. Hepatitis B viral loads can increase markedly following allogeneic stem cell transplantation in chronically infected patients, and there is a high risk of fulminant hepatitis at the time of immune recovery, although the risk can be reduced by the use of antiviral agents such lamivudine (Francisci et al., 2006).

1.3.2 Non-infectious Complications

Non-infectious complications are generally the result of GVHD, complications of the conditioning regimen or immunosuppressive agents, or a complication of drugs used to treatment infection. All organ systems

of the body may be affected following transplantation. Although by no means an exhaustive list, some of the more common complications are listed in Table 1-1.

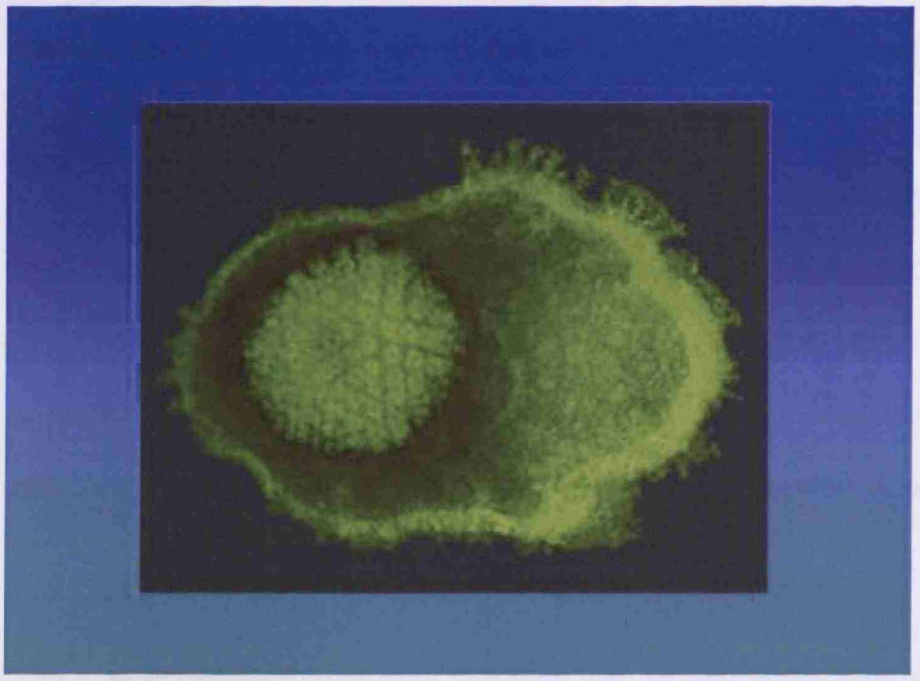
Table 1-1 Major non-infectious complications of allogeneic stem cell transplantation.

Organ System	*Complication*	*Cause*
Pulmonary	Idiopathic Pneumonitis	
	Diffuse Alveolar Haemorrhage	Thrombocytopaenia
	Bronchiolitis Obliteraans	GVHD
Cardiac	Cardiomyopathy	Cyclophosphamide, prior Anthracyclines
	Arrhythmias	
	Cardiac Tamponade	Thalassaemia
Renal	Drug Nephrotoxicity	Ciclosporin, Aminoglycoside, Amphotericin
	BMT-related Thrombotic Microangiopathy	HLA mismatched transplants
Hepatic	Veno-occlusive Disease	High dose busulfan
	Liver GVHD	HLA mismatched transplants
Gastro-intestinal	Gut GVHD	HLA mismatched transplants
	Mucositis	TBI, methotrexate
Neurological	Metabolic Encephalopathy	Ciclosporin
	Seizures	Busulfan, Ciclosporin
	Leukoencephalopathy	Intrathecal methotrexate, cranial irradiation
Haematologic	Immune Thrombocytopaenia	
	Autoimmune Hemolytic Anaemia	
	Alloimmune Hemolytic Anaemia	ABO incompatibility
Endocrine	Hypothyroidism	TBI
	Hyperthyroidism	
Gynaecologic	Infertility	Radiation, high dose chemotherapy
Ocular	Cataracts	TBI

1.4 Discovery of Human Cytomegalovirus

The first description of the histopathological changes characteristic of HCMV infection was made in 1904 by Jesionek from an autopsy study of a premature infant thought to have died of syphilis (Jessionek, 1904). He described enlarged cells, 20-30 μm in diameter, with intra-nuclear inclusions but attributed the changes to a protozoa infection. The term 'cytomegali' was applied to these histological features by Goodpasture and Talbot in 1921 (Goodpasture and Talbot, 1921). In 1926 Cole demonstrated that these histological changes could be induced in guinea pigs by a transmissible agent, and speculated that it was most likely a virus (Cole and Kuttner, 1926). In 1956 the HCMV virus was independently isolated by three groups (Rowe et al., 1956; Smith, 1956; Craig et al., 1957), with the name cytomegalovirus being applied by Weller's group because of the effect of viral infection in cell culture. An electron micrograph image of the HCMV virion, illustrating the capsid, tegument and envelope with surface glycoproteins is shown in Figure 1.1.

Figure 1-1 Electron micrograph image of the HCMV virion (Courtesy of Vincent Emery).



1.5 Classification of Human Cytomegalovirus

HCMV is a member of the family Herpesviridae, subfamily Betaherpesviridae, genus Cytomegalovirus, and species Human herpesvirus 5. The family herpesviridae is characterised by a linear double stranded DNA, an icosadeltahedral capsid, a tegument and an envelope embedded with glycoprotein spikes. Based on biological properties, the herpesviridae family is subdivided into three groups, the alphaherpesviridae, the betaherpeseviridae and the gammaherpesviridae (Roizman, 1995). HCMV, along with Human herpesvirus 6 and human herpesvirus 7, belong to the betaherpesviridae subfamily, which is characterised by a slow replication in culture, cytomegalia formation, and latency in secretary glands, lymphoreticular cells, kidneys and other tissues.

HCMV displays many sites of genetic variability and can be subdivided into identifiable strains based on genotyping. However, infection with one strain of HCMV does result in a level of immunity to other strains (Chandler and McDougall, 1986). The sequence for the gene encoding the gB protein has been used to subdivide HCMV into 4 genotypes (Chou and Dennison, 1991). Although useful for epidemiological studies, the evidence that different genotypes have an influence on virulence and clinical outcome is contradictory and inconclusive (Pignatelli et al., 2004).

1.6 Epidemiology of Human Cytomegalovirus

The overall prevalence of prior infection with HCMV in the general population is 70 percent for countries with good socio-economic status, and 90 percent for those countries with a poor socio-economic status (Pass, 1985). The virus is most commonly acquired during childhood, frequently from other children as demonstrated by the increased frequency of infection among children attending day care centres (Pass et al., 1984). Infection as an adult may occur through sexual contact (Handsfield et al., 1985) or acquired from infected children as evidenced by the increased rate of infection among adult workers in childcare facilities (Pass et al., 1990). HCMV can be found in saliva, semen and cervicovaginal secretions, and in children the virus may be excreted in the urine for many months following primary infection (Gehrz et al., 1982). Vertical transmission occurs via the transplacental route or intrapartum from cervical secretions present at birth, as well as via human breast milk postpartum. The HCMV transmission rate to neonates of seropositive mothers is 2.2 percent, and in mothers experiencing primary infection

during gestation is as high as 20 to 40 percent (Griffiths and Baboonian, 1984). Nosocomial transmission may occur as a result of receiving an HCMV infected solid organ or stem cell graft or by a blood transfusion, although the later risk is much reduced since the adoption of universal leukodepletion (de Witte et al., 1990).

1.7 Human Cytomegalovirus Structure and Organisation

1.7.1 Structure

At 300 nm in diameter, HCMV is the largest of the herpes viruses. It consists of a lipid envelope spiked by immunogenic glycoproteins and an icosadeltahedral capsid made up of 162 capsomeres enclosing the DNA. By electron cryomicroscopy at a resolution of 18 Angstrom it has been shown that the capsid proteins form an icosahedral structure consisting of 12 pentons, 150 hexons and 320 triplexes (Chen et al., 1999). Between the envelope and the capsid is the tegument, an electron dense layer containing proteins important for controlling host cell function following viral entry into the cell (Spaete et al., 1994; Britt and Mach, 1996; McGavran and Smith, 1965). As well as the complete virus, two non-infectious forms can be identified on electron microscopy: the dense body which is an amorphous structure without a capsid or DNA, and the non-infectious enveloped particle, consisting of an empty capsid (Roby and Gibson, 1986).

1.7.2 Genomic Organisation

The HCMV genome is organised into two linked segments (L and S). Each segment is made up of a unique sequence (U_L and U_S) flanked on either side by an inverted repetitive segment (TR_L, IR_L, TR_S, IR_s), and the segments can be arranged into four concatameric forms (Bankier et al., 1991).

The first complete genome sequence for HCMV was described for the highly passaged AD169 strain, a laboratory strain of HCMV initially developed as a vaccine candidate (Elek and Stern, 1974). The genome length is reported as 235 kilobase pairs, and was originally reported to encode 208 non overlapping Open Reading Frame's (ORF), with 14 duplications (Chee et al., 1990). Recent work using sophisticated gene finding algorithms has attributed only 192 ORFs to the AD169 strain (Murphy et al., 2003a). Compared to low passaged (less then 4) strains such as Merlin and Toledo, 22 open reading frames are missing from the AD169 sequence (Dolan et al., 2004; Cha et al., 1996). Thomas Shenk's team at Princeton University has recently compared the sequences of AD169 and Towne HCMV strains to the Toledo and clinical HCMV strains (isolated from a bone marrow transplant patient, a pregnant woman with primary HCMV infection and an HIV patient with retinitis) (Murphy et al., 2003b). By using bacterial artificial chromosomes and extensive sequencing, they have shown that clinical HCMV strains contain 252 ORFs, including an additional sequence between UL133 to UL151 and an additional IR_L sequence. A map of the organisation of the HCMV genome from a clinical HCMV isolate is shown in Figure 1-2. Many of the missing

genes from laboratory strains of HCMV encode immuno-evasion genes and are dispensable for growth in cell culture (Kollert-Jons et al., 1991), such as the chemokine analogues UL146 and UL147 (Prichard et al., 2001).

Figure 1-2 Map of the HCMV genome, reproduced with permission from Thomas Shenk (Murphy et al., 2003a)

1.7.3 Important Structural Proteins

The ORFs of AD169 plus additional missing sequences found in the Toledo and Towne strains are predicted to encode at least 213 proteins (Novotny et al., 2001). Using 'in silico' or automated pattern based computer algorithms to elucidate protein function, the HCMV genome encodes a large number of glycoproteins, including 125 ORFs with glycosylation sites (Rigoutsos et al., 2003), much greater then the 53 originally identified by Chee (Chee et al., 1990). In addition, 144 integral membrane proteins (half of which are glycoproteins), nine phosphorylation sites, and 15 ORFs with G-protein sequences have been identified (Rigoutsos et al., 2003). Although not all the genes encoded by HCMV have had functions attributed, the following is a brief description of the more important structural proteins. Genes regulating transcription and viral DNA replication are discussed in section 1.8.2 (Viral Replication), and the genes involved in immuno-evasion are discussed in section 1.10 (Immune Manipulation by HCMV).

Capsid Proteins

The major capsid protein (MCP, UL86) is the most abundant capsid protein. Five copies form the capsid penton, and six copies in association with six copies of the smallest capsid protein (SCP, UL48.5) form the capsid hexon (Chen et al., 1999). Two copies of the minor capsid protein (mCP, UL85) and one copy of the mCP binding protein (mCP-BP, UL46) form the triplex. In addition fragments of the two scaffolding proteins, the

maturational protease precursor, pUL80a, and the assembly protein precursor, pUL80.5 are also contained within the capsid (Loveland et al., 2007).

Tegument proteins

The viral tegument contains at least 25 proteins, and many of these proteins have a role in HCMV replication by regulating viral gene expression or modulating the host cell. The tegument proteins are mainly phosphoproteins, (denoted by the suffix 'pp'), are highly immunogenic, and are important for the early stages of gene transactivation and the taking over of the host's cellular machinery following penetration. Included among this group is pp65 (encoded by UL83 and also known as lower matrix protein), the most abundant tegument phosphoprotein. It is a major target for both the humoral (Jahn et al., 1987) and cellular immune response (Bitmansour et al., 2001; Kern et al., 2002; Laughlin-Taylor et al., 1994). Following cell penetration by HCMV, pp65 rapidly translocates to the cell nucleus (Schmolke et al., 1995a), and plays a role in immune modulation (see section 1.10). The tegument phosphoprotein pp71 (encoded by UL82 and also known as the upper matrix protein), transactivates immediate early viral promoters and is a cell cycle regulator, targeting the cellular Retinoblastoma family of tumour suppressor proteins for degradation, inducing DNA synthesis in otherwise quiescent cells (Kalejta et al., 2003). Acting together with pp71 is pUL69, another immediate-early viral transactivator, which has also been shown to arrest cell cycle progression in G1 phase (Lu and Shenk, 1999). PP150 (encoded by UL32, and also known as basic phosphoprotein) is the

second most abundant phosphoprotein in the capsid and is also highly immunogenic (Jahn et al., 1987; Gyulai et al., 2000; La Rosa et al., 2005). The UL84 gene product, pp86 (IE2) is a transactivator of both viral and cellular genes (Gebert et al., 1997), while UL26 is another important transcriptional activator (Stamminger et al., 2002).

Envelope proteins

Envelope proteins are important for viral binding to the cell surface and are a major target for neutralizing antibodies in immune human serum. To date glycoprotein B (gB), glycoprotein H (gH), glycoprotein L (gL) and glycoprotein M (gM) have been shown to be essential for production of infectious virus (Hobom et al., 2000). The most abundant envelope protein is gB (encoded by UL55), with 906 amino acid residues and constitutes the disulfide-linked glycoprotein complex I (gCI) (Kari et al., 1990). Transcribed in the early and late phase of viral replication, this large immunogenic protein protrudes from the envelope and is important for adsorption, cell fusion and the cell-to-cell spread of HCMV (Tugizov et al., 1994). The second disulfide linked glycoprotein complex is gCII, and is comprised of gM (encoded by UL100) and gN (encoded by UL73) (Mach et al., 2000), while the third glycoprotein complex, gCIII, is formed from gH, gL as well as glycoprotein O (Huber and Compton, 1998; Li et al., 1997).

1.8 Human Cytomegalovirus Infection

1.8.1 Tissue tropism

In vivo, HCMV infects fibroblasts, epithelial cells, macrophage, monocytes, smooth muscles cells, and endothelial cells. In culture, the AD169 strain of HCMV displays tropism for human fibroblast cell lines, although clinical HCMV isolates have been shown to infect endothelial cells (Sinzger et al., 1999b; MacCormac and Grundy, 1999), hepatocytes (Sinzger et al., 1999a) and cells of the monocyte/macrophage lineage (Fish et al., 1996; Taylor-Wiedeman et al., 1994) . Following passage in cell culture, the HCMV genome undergoes loss of some of the genetic content important for virulence and immuno-evasion, but dispensable for viral replication (Mocarski, Jr., 2002).

1.8.2 Viral Replication

Cell Entry

Substantial progress has recently been made into the means by which HCMV gains entry into the cell. Glycoprotein B in concert with a complex of the glycoproteins gH, gL and gO, binds to epidermal growth factor receptor (EGFR) and the integrins, alpha2beta1, alpha6beta1 and alphavbeta3 act as coreceptors, to stimulate viral entry (Boyle and Compton, 1998; Feire et al., 2004; Wang et al., 2005; Wang et al., 2003). This triggers a cell signalling cascade similar to the effect of cell stimulation with interferon gamma (Simmen et al., 2001). By fusion of the virus envelope with the cell membrane, the virus penetrates the cell

membrane, gaining entry into the cytoplasm and the nucleocapsid then makes its way to the nucleus.

Immediate Early, Early and Late Gene Expression

There is a clearly defined sequence of protein expression in cells infected by HCMV (Stinski, 1978). Immediate early expressed genes are those genes expressed on viral entry and do not require the expression of other viral genes (Spector, 1996). Many are transactivators for DNA replication. The most important are IE1 (also known as IE72) and IE2 (also known as IE86), generated through differential splicing and polyadenylation of a primary transcript of the major immediate early (MIE) region, consisting of the ORFs UL122 and UL123. They play a central role in controlling viral and cellular gene transactivation in order to facilitate viral DNA production (Stenberg, 1996). Both IE1 and IE2 further enhance major immediate early (MIE) gene transcription through NF-kappaB mediated binding of the enhancer region of MIE and IE2 autoregulates its own promoter by binding to the cis repression sequence. Other immediate early genes are also known to play a role (Colberg-Poley, 1996). Early gene expression is dependent on the expression of immediate early gene products, and includes mainly viral DNA replication genes such as UL54. Late gene expression includes many of the structural proteins gB and gH. The total transition from immediate early to late phase takes 24 to 36 hours, with maximal virus production at 72 to 96 hours.

Taking Over of Cell Machinery and Arresting Cell Division in S Phase

HCMV is not capable of producing its own RNA, and therefore must take over the machinery of the cell and subvert it for its own purpose. Three genes have been identified as being responsible for manipulating the cell cycle. The ppUL69 blocks the cell at the G1 phase (Hayashi et al., 2000; Lu and Shenk, 1999). The UL82 gene product, pp71, stimulates the cell cycle through G1 phase (Kalejta and Shenk, 2003). IE1 inhibits cell cycle progression from G1 to S phase by activation of a p53 response (Castillo et al., 2005).

In addition, HCMV has also evolved a number of mechanisms to inhibit apoptosis. UL122 and UL123 block apoptosis by an as yet unknown mechanism (Zhu et al., 1995). The protein pUL36 (viral inhibitor of caspase-8 induced apoptosis, vCIA) inhibits FAS mediated apoptosis by blocking the cleavage of procaspase-8 (Skaletskaya et al., 2001; Zhu et al., 1995). The protein pUL37 (viral mitochondrial-localized inhibitor of apoptosis, vMIA) acts as a mitochondrial inhibitor of apoptosis by interfering with the adenine nucleotide translocase Bax, which controls cytochrome C release during apoptosis (Poncet et al., 2004).

Assembly of New Infectious Units

Replication and assembly of the new virion occurs in the host cell nucleus. Six HCMV proteins constitute the replication fork responsible for viral genomic replication, a two sub-unit DNA polymerase consisting of pUL54 and its associated protein ppUL44, a three sub-unit helicase-

primase complex made up of pUL70, pUL102, and pUL105, and a single stranded DNA binding protein, ppUL57 (Anders and McCue, 1996). The viral genome is produced by rolling circle replication, with initial circularization of the parental genome followed by the production of concatemers of viral DNA, with the addition of the genomic termini at the cleavage and packaging stage (McVoy and Adler, 1994). The constituent capsid proteins form an icosahedral procapsid structure through protein interaction and self-assembly, with the proteins UL80a and UL80.5 providing a scaffolding function (Loveland et al., 2007). The viral DNA probably enters the capsid through a single portal in a mechanism similar to that demonstrated using time lapse cryo-electron microscopy for herpes simplex virus capsid assembly (Cardone et al., 2006; Heymann et al., 2003). The putative HCMV portal protein is pUL104, a protein which co localizes both in the nucleus and cytoplasm with the HCMV terminase protein pUL56, which is responsible for cleaving the viral DNA into genome length units (Dittmer et al., 2005). In common with other herpes viruses, it is likely that following initial capsid formation within the nucleus, the nucleocapsid enters the cytoplasm by envelopment at the inner nuclear membrane, and then de-envelopment occurs at the outer nuclear membrane. Final envelopment prior to release of the virus occurs at the trans-Golgi network, where the major glycoproteins are assembled (Crump et al., 2003). Evidence for the so called re-envelopment model (Smith, 1980) has been supported by recent immunoflorescence and cell tracking studies (Turcotte et al., 2005).

HCMV can spread from cell to cell by direct contact and fusion with neighbouring infected cells, as demonstrated by the transfer of green fluorescent labelled HCMV protein from infected to uninfected cells (Digel et al., 2006) . When endothelial cells are infected with wild type but not laboratory HCMV strains, culturing with polymorphonuclear cells results in transitory micro fusion events (Gerna et al., 2000), resulting in the transfer of infectious virus to leucocytes which can then disseminate to sites distant to the primary infection. Supporting evidence for dissemination by infected leukocytes is provided by the fact that the quantity of HCMV present in the leukocyte fraction is higher than plasma or cell free component (Gerna et al., 1994), and infectious virus can only be cultured from the cellular compartment (Lipson et al., 2001).

1.8.3 Latency and Reactivation

Viral latency has been defined as "the persistence of viral genome in the absence of production of infectious virions, but with the ability of the viral genome to reactivate after specific stimuli" (Sinclair and Sissons, 2006). In healthy HCMV seropositive individuals, using sensitive PCR techniques, peripheral blood monocytes are the major site for HCMV DNA (Larsson et al., 1998). It is likely that this is the site of HCMV latency, as even highly sensitive reverse transcriptase PCR (RT-PCR) fails to detect productive viral IE gene products in monocytes and monocyte precursors of seropositive HCMV carriers (Taylor-Wiedeman et al., 1994). HCMV DNA has also been demonstrated in bone marrow derived CD34+ myeloid progenitors (Mendelson et al., 1996). The exact mechanism by which HCMV maintains latency is not clear, but latency

associated transcripts have been identified in granulocyte macrophage precursors arising from alternative expression of the MIE gene locus, including a sense transcript with two novel start sites and an unspliced anti-sense transcript complementary to IE1 exons 2-4 (Kondo et al., 1996).

Reactivation from latency can occur as a result of immunosuppression (Reinke et al., 1999), and as a result of the cytokines released as part of the alloreactive process occurring during GVHD reactions (Soderberg-Naucler et al., 1997). Certain pro-inflammatory cytokines such as interferon gamma, tumour necrosis factor, interleukin four and granulocyte macrophage colony stimulating factor (GM-CSF) can stimulate reactivation of latently infected CD33+ cells into active viral replication in vitro (Hahn et al., 1998).

1.9 Role of the Immune Response in Controlling Human Cytomegalovirus Infection

Both the innate and adaptive immunity are important for controlling HCMV infection. Following initial inoculation, the virus infects epithelial cells of the mucosal linings. This results in an inflammatory response, with recruitment of neutrophils, which then disseminate the infection. At this time, the infected individual becomes viraemic. In otherwise healthy individuals, the innate immune, humoral and cellular immune response acts to contain the infection and maintain the virus in a latent state.

1.9.1 Innate Immune Response

Toll Like Receptors

Through the interaction of gB and gH on the viral envelope with the Toll like receptors TLR1 and TLR2 on the host cell surface, activation of the cells innate immune system occurs, with the induction and secretion of inflammatory cytokines via the NF-kappaB pathway (Boehme et al., 2006; Compton et al., 2003). Toll like receptors are a family of receptors that recognize microbial pathogens, and stimulate the innate immune system when they recognize pathogen associated molecular patterns (PAMP's). It is conceivable that HCMV may utilize inflammatory cytokine release and subsequent recruitment of neutrophils and macrophages to disseminate infection and facilitate viral replication.

Natural Killer Cells

Mouse strains deficient in natural killer (NK) cells are at increased susceptibility to murine CMV infection and disease (Shellam et al., 1981). In humans, NK cells are capable of killing HCMV infected cells in an interferon gamma independent manner (Borysiewicz et al., 1985), and congenital deficiency of NK cells results in susceptibility to HCMV infection (Biron et al., 1989). The fact that HCMV has evolved a large number of mechanisms to evade NK cell killing (see section 1.10) further also testifies to the importance of the NK cell response. Following allogeneic stem cell transplants, NK cell counts tend to be higher in HCMV seropositive than HCMV seronegative recipients (Hokland et al., 1988; Kook et al., 1996). Recently it has been shown that in allogeneic

stem cell transplants where both the donor and the recipient are HCMV seropositive, a donor with more than one activating killer immunoglobulin-like receptor (KIR) was associated with a 65 percent reduction in HCMV infection (Cook et al., 2006).

1.9.2 Humoral Immune Response

Following initial infection with HCMV, a primary IgM response occurs, which gradually matures over 6 weeks until an HCMV specific IgG response develops. The major HCMV targets of human serum are the surface glycoproteins gB, gH, gM, gN, and the phosphoproteins pp150 and pp52 (Schoppel et al., 1997; Schoenberger et al., 1998). Between 40 and 70 percent of the humoral response to HCMV is directed to the glycoprotein gB (Britt et al., 1990). There is evidence that although the humoral response is not essential for resolution of primary HCMV infection, it limits dissemination of recurrent virus (Schoppel et al., 1997). The humoral response to HCMV appears to provide protection from HCMV disease in the neonate (Yeager et al., 1981) and the solid organ setting (Snydman and Falagas, 1996).

1.9.3 Cytotoxic T Cell Response

CD8+ T cell recognise peptides presented by Class I HLA molecules present on all cells in the body. Antigens presented by Class I molecules are derived from the proteosomic degradation of the proteins of intracellular pathogens including HCMV. The resultant peptides are then transported to the lumen of the endoplasmic reticulum by TAP (transporter associated protein), and loaded onto Class I molecules by

tapasin. HLA Class I molecules bind peptides of 8-10 amino acids at both ends, while interaction between anchoring residues of the peptide and the binding groove of the HLA molecule determine specificity (Rammensee, 1995). For instance the pp65 peptide, NLVPMVATV has been identified as an HLA A*0201 restricted HCMV specific epitope (Diamond et al., 1997; Solache et al., 1999). The Class I heavy chain, $\beta_2$microglobulin, and the antigenic peptide form a stable complex which is then transported to the cell surface, ready for antigenic recognition by CD8+ T cells. Following an acute infection, there is an initial expansion of antigen specific CD8+ cytotoxic T-cells (CTLs), which acquire effector functions including: the ability to secrete tumour necrosis factor alpha (TNFα) and interferon gamma (IFGγ), express cytotoxic granules such as perforin and granzyme, and the ability to enter non-lymphoid tissue. Two to three weeks after maximal expansion, 90 to 95 percent of these cells undergo apoptosis. A subset of these cells acquire a memory phenotype, characterised by the ability to be maintained in the absence of an antigen, and with stimulation reacquire the ability to produce TNFα and IFGγ as well as cytotoxic function, and with maturation increased capability to secrete interleukin 2 (IL2) . Two types of memory cells can be identified: effector memory (T_{EM}) cells characterised by $CCR7^{low}$ and $CD62L^{low}$ expression and an inability to enter lymph nodes via high endothelial venules, and central memory (T_{CM}) cells characterised by $CCR7^{high}$ and $CD62L^{high}$ expression and the ability to enter lymph nodes and lymphoid organs such as the spleen (Sallusto et al., 1999). Both cell types produce TNFα and IFGγ but only T_{CM} cells produce high levels of IL2. Over time,

there is a transition from T_{EM} to T_{CM}. With antigenic restimulation, T_{CM} cells revert to T_{EM} cells. In the presence chronic viral infections, such as HCMV, a number of changes in the immune response occur (Gamadia et al., 2001). Over time, the CTL memory response becomes dominated by a small number of greatly expanded clones responding to a few peptides, and characterised by a CD57+ CD28- CD8+ phenotype (Weekes et al., 1999). There is also a reversion from $CD45RO^{high}$ to $CD45RA^{high}$ in vivo. The main targets of the CTL response are IE1 and pp65 (Kern et al., 1999; Khan et al., 2002a), with pp65 dominating (Wills et al., 1996; Laughlin-Taylor et al., 1994), although responses to pp150 are also significant (Gyulai et al., 2000; La Rosa et al., 2005). Class I tetramer technology (Altman et al., 1996) has greatly facilitated the study of antigen specific T-cells. The CD8 response to HCMV has one of the highest CD8+ T cell antigen specific frequencies recorded, ranging from 1 percent in healthy individuals up to 10 percent of the total CD8 T cell population in BMT and organ transplant recipients (Emery, 2000; Engstrand et al., 2000; Gillespie et al., 2000; Singhal et al., 2000). With increasing age the frequency of HCMV specific CD8+ T cell frequency continues to increase, although there is evidence that this is also associated with impaired function, suggesting immune burn-out due to chronic stimulation (Ouyang et al., 2003).

1.9.4 Helper T Cell Response

Following primary infection, activation of naïve CD4 T cells occurs through recognition of foreign peptides on HLA class II molecules on the surface of professional antigen presenting cells (APCs), such as dendritic

cells, macrophages and B cells. Degradation of endocytosed proteins within endosomes or lysosomes generates the peptides presented by Class II HLA molecules. The Class II α and β heavy chains are stabilised in the endoplasmic reticulum by the invariant chain, which blocks the binding groove, and the complex is then transported to endocytic vesicles, where the invariant chain is broken down and peptide loading occurs. Although anchoring residues are also important in determining peptide affinity for Class II molecules, peptide binding conditions are more relaxed than Class I, with a minimum of 12 amino acids, but not constrained by a maximum length, with the ends of the binding groove being open. After being transported to the cell surface, the peptide loaded class II molecule, in conjunction with the costimulatory CD28 ligand B7, interacts with the T-cell receptor and its co-receptor CD4, to activate the T cell. After activation, naïve CD4+ T cells differentiate into either T_H1 cells, characterised by IFγ secretion and protection against intracellular infection, and T_H2 cells, characterised by IL4 secretion and protection against extracellular infections (Seder and Ahmed, 2003). Like CD8+ T cells, the majority of the initial expansion of CD4+ activated cells undergoes apoptosis. Some of the T-cells stimulated in the T_H1 direction develop into long-term memory cells. Naïve CD4+ T-cells express CD45RA, $CD62L^{HI}$ and are $CCR7^-$. T_{CM} cells are characterized by a $CD45RA^-$, $CD62L^{HI}$, and $CCR7^+$ phenotype, while T_{EM} cells have a $CD62L^{Hi/Lo}$, $CCR7^-$, and $CD45RA^{+/-}$ phenotype.

CD4 T helper cells play a number of crucial roles in the immune response to viral infections:

i) Mediate the release of inflammatory cytokines such as IL2 (Su et al., 1998).

ii) Condition the antigen presenting cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

iii) Have direct cytotoxic activity on virally infected cells (Appay et al., 2002).

iv) Provide help for virus specific antibody production (Jonjic et al., 1994).

Animal models have demonstrated that the function of memory CD8 T cells is impaired in the absence of CD4 help (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). In humans infected with chronic viral infections, CD4+ T cells play a crucial role in sustaining CTL responses (Matloubian et al., 1994), as well as the secondary expansion and development of memory CD8+ T cells, (Schoenberger et al., 1998). It has been suggested that CD4+ T cells, through CD40L, may imprint a unique molecular signature on effecter CD8+ T cells, giving them the ability to improve memory function (Bourgeois et al., 2002), perhaps by conditioning the APC via interaction of CD40L with CD40 (Janssen et al., 2003).

Proliferative HCMV specific CD4+ T cells responses have been demonstrated to pp65, gB, gH, IE1, IE2, UL69, pp71, and gpUL18 with the dominant responses to pp65 (Beninga et al., 1995; He et al., 1995). Based on antigen specific cytokine production, 63 percent of healthy donors have a detectable CD4 response to IE1 (Kern et al., 2002).

In addition, a subset of cytotoxic CD4 T cells express perforin and granzyme B, are characterised by the absence of the costimulatory molecules CD28 and CD27 (Appay et al., 2002), and are described in chronic viral infection. These cells have been described following primary HCMV infection with proliferative response specifically to HCMV (van Leeuwen et al., 2004; van Leeuwen et al., 2006). Further evidence for the importance of CD4 helper function in controlling HCMV infection is that HCMV disease in HIV patients normally only occurs when the CD4 count falls below 50 X 10^6/L (MacGregor et al., 1995). Recovery of HCMV specific immunity with HAART therapy is correlated with a fall in the incidence of HCMV infection and HCMV disease (Komanduri et al., 1998).

1.10 Immune Manipulation by HCMV

Human CMV has probably evolved more ways to evade and modify the immune system then any other human viral pathogen, perhaps reflecting the fact that beta-herpes viruses have been co-evolving with their hosts for at least 180 million years (McGeoch et al., 1995). These mechanisms are likely to be responsible for the enormous success of this pathogen in achieving such a high prevalence and maintaining lifelong latency without apparently harming the host. Much of the initial investigation into the strategies by which CMV evades the immune system was performed in the mouse , as murine CMV uses many of the same immunoevasion mechanisms as HCMV, and this is well reviewed by Reddahase (Reddehase, 2002; Reddehase et al., 2004). Table 1-2 summarises the mechanisms by which CMV evades the immune system in the human

host, although this list is not comprehensive and undoubtedly, further strategies will continue to be uncovered.

Down regulation of MHC class I and II

HCMV is capable of modification of the HLA pathways through the expression of immunoevasion genes, which result in the down regulation, decreased expression and increased degradation of both HLA class I and class II molecules. The expression of these genes is also dependent on the stage of viral replication with different genes being active at different stages. The US2 to US11 region encodes four glycoproteins involved in the down regulation of HLA class I expression. The gene product of US3, gpUS3, expressed during the immediate early phase of replication, inhibits tapasin mediated peptide loading in the endoplasmic reticulum by impairing HLA Class I heavy chain egress from the endoplasmic reticulum (Jones et al., 1996). US2 and US11, which are both expressed in the early phase, down regulate HLA class I by redirecting the nascent HLA class I protein from the endoplasmic reticulum to the cytosol (Story et al., 1999). US6, which is expressed in the early and late phase, blocks TAP in the endoplasmic reticulum (Ahn et al., 1997). In vitro studies have demonstrated that all four gene products are individually capable of down regulating HLA class I expression. In addition to their effect on levels of HLA class I expression, US3 can down regulate HLA class II expression by disrupting the invariant chain interaction (Hegde et al., 2002), and US2 by inhibiting HLA class II translocation (Tomazin et al., 1999). Infection by HCMV of the professional antigen presenting cell, the dendritic cell

has been shown to down regulate HLA class II antigen presentation (Jahn et al., 1999).

Avoidance of NK cell killing

The reduction of HLA class I surface expression would normally render a cell vulnerable to NK cell killing, due to the absence of an inhibitory signal. However, HCMV has also evolved a remarkable array of strategies to bypass NK cell lysis. UL141 blocks the surface expression of CD155, the ligand for NK cell activating receptors CD226 and CD96 (Tomasec et al., 2005), and UL142 encodes an HLA class I like molecule that down regulates the NKG2D ligand, MICA (Chalupny et al., 2006; Wills et al., 2005). UL16 is responsible for the down regulation of the NKG2D ligands MICB and UL binding proteins (Vales-Gomez et al., 2006). HLA-E normally presents the leader sequence of other Class I HLA proteins at the cell surface, and inhibits NK cell activation by binding to the CD94/NKG2A receptor. The leader sequence of gpUL40 can up regulate HLA-E independent of TAP, as demonstrated by up regulation of HLA-E expression in HCMV infected TAP deficient cells, thereby inhibiting NK cell mediated cell lysis (Ulbrecht et al., 2000; Tomasec et al., 2000). UL18 complexes with human β2-microglobulin and is expressed on the surface as a HLA Class I homologue. Paradoxically, it has been shown to increase NK killing, but it is also binds to the leukocyte immunoglobulin like receptor (LIR1) on macrophages, acting as a decoy (Chapman et al., 1999).

Cytokine and cytokine receptor analogues

HCMV encodes a viral homologue of interleukin 10 (IL10), cmvIL10, encoded on UL111a (Kotenko et al., 2000). This chemokine homologue has affinity for both IL10R1 and IL10R2 receptors. It has a number of immunosuppressive properties, including: inhibition of mitogen stimulated monocyte proliferation, inhibition of cytokine secretion, down regulation of HLA class I and II molecules, blocking of cytokine induced differentiation of monocytes into functionally active phagocytic macrophages and inhibition of dendritic cell maturation and function (Chang et al., 2004; Gredmark et al., 2004; Spencer et al., 2002). UL146 and UL147 encode CXC chemokines. The UL146 gene product is a viral CXC chemokine that can bind to the CXCR2 IL8 receptor, and shows equal potency to human IL8 as a neutrophil chemoattractant (Penfold et al., 1999; Saederup and Mocarski, Jr., 2002). There is considerable sequence variation in UL146 and UL147, and based on studies of congenitally infected infants there is some early evidence that this may contribute to differences in disease manifestations (He et al., 2006; Lurain et al., 2006; rav-Boger et al., 2006). US28 encodes an HCMV CC chemokine receptor which sequesters and internalizes the chemokines monocyte chemotactic protein 1 (MCP1) and RANTES surrounding an infected cell, preventing leukocyte chemotaxis and activation of effecter function, as well as initiating cell signalling pathways in infected cells (Billstrom et al., 1998; Bodaghi et al., 1998).

Subversion of Innate Cellular Immunity

The tegument phosphoprotein UL71 subverts a cellular innate immune defence mechanism, promyelocytic leukaemia nuclear body (PML-NB) protein Daxx, which would otherwise block immediate early gene expression through histone deacetylase activity, by mediating its proteosomal degradation (Saffert and Kalejta, 2006). The phosphoprotein pp65, by interfering with NF-kappaB and interferon response factor I and 3 (Abate et al., 2004), inhibits the ability of cells to respond to exogenous interferon (Browne and Shenk, 2003). pp65 also has the ability to phosphorylate IE1, thereby preventing its breakdown in the proteosome, and subsequent presentation by HLA class I molecules (Gilbert et al., 1996). Despite these roles, pp65 is in fact dispensible for cell growth in culture (Schmolke et al., 1995b).

Table 1-2 Mechanisms by which HCMV evades host immune system

Mechanism		*Reference*
Down Regulation of MHC Class I and II		
US2	Redirects MHC Class I proteins from ER to cytosol	(Story et al., 1999)
	Inhibit MHC Class II by degrading HLA DR-alpha and DM-alpha	(Tomazin et al., 1999)
US3	Impairs MHC Class I heavy chain egress from ER	(Jones et al., 1996)
	Down regulates MHC Class II by disrupting invariant chain interaction	(Hegde et al., 2002)
US6	Down regulates MHC Class I by blocking TAP in ER	(Story et al., 1999)
US11	Redirects MHC Class I proteins from ER to cytosol	(Ahn et al., 1997)
Avoidance of NK Cell Killing		
UL16	Down regulates the NKG2D ligands MICB and UL binding protein	(Vales-Gomez et al., 2006)
UL40	Up regulates cell surface HLA-E expression	(Ulbrecht et al., 2000) (Tomasec et al., 2000)
UL141	Blocks surface expression of CD155, ligand for CD226 and CD96	(Tomasec et al., 2005)
UL142	Encodes MHC Class I like molecule that down regulates the NKGSD ligand MICA	(Wills et al., 2005)
Cytokine and cytokine receptor analogues		
UL111a	cmvIL10, a viral analogue of IL10	(Kotenko et al., 2000)
UL146	viral CXC chemokine 1, a ligand for the CXCR2 IL8 receptor	(Penfold et al., 1999)
UL147	viral CXC chemokine	(Penfold et al., 1999)
US28	cmv CC chemokine receptor, neutralizes MCP1 and RANTES	(Billstrom et al., 1998) (Bodaghi et al., 1998)
Subversion of innate cellular immunity		
UL71	Proteosomal degradation of Daxx	(Saffert and Kalejta, 2006)
UL83 (pp65)	Inhibition of interferon response via NFkapppaB and Interferon Response Factor 1	(Abate et al., 2004)
	Phosphorylation of IE1, preventing proteosomal degradation and MHC Class I presentation	(Gilbert et al., 1996)

1.11 Immune Recovery Following Allogeneic Stem Cell Transplantation

Recovery of the immune system following stem cell transplantation includes recovery of the innate immune system, B cell immune function, and T-cell immune function.

The innate immune system is the first component to recover. Injury sustained to the mucosal barrier due to the conditioning regimen takes between one and three months to heal, and during this time is a significant portal of entry for infections, particularly bacterial pathogens. Neutrophil counts normally recover within a month following transplantation, but the recovery of neutrophil functional deficits such as chemotaxis, superoxide production and phagocytic activity takes significantly longer (Zimmerli et al., 1991). NK cell recovery, which is important for the control of HCMV infection, is detectable as early as one week following peripheral blood stem cell transplantation, with similar frequencies to normal donors at 3 months, although absolute levels remain less then normal donors even at 12 months following transplant (Chklovskaia et al., 2004).

B cells recovery is important for reconstitution of the humoral immune system. B cell levels remain undetectable for one to two months post transplantation, but by 1-2 years, they rise to greater then normal levels, with non-myeloablative transplants being associated with slower recovery (Petersen et al., 2003). B cell recovery probably occurs due to regeneration of naïve B cells from the donor stem cell, rather then

infusion of the mature B cells from the graft. Serum levels of immune globulins fall immediately post transplant. Apart from responses to antigens encountered early post transplant, such as the HCMV proteins glycoprotein gB, other common antigen specific antibody levels gradually fall post transplant (Engelhard et al., 1991; Lutz et al., 1996). The non-specific antibody levels of IgM, IgG1 and IgG3 recover to normal or greater then normal levels within months, but IgA and IgG4 can take several years, while the IgG2 subclass is the last to recover (Sullivan et al., 1996). Antibody specificity post transplant is of a limited spectrum but may include significant numbers of auto antibodies, particularly in the presence of chronic GVHD (Patriarca et al., 2006).

The cellular immune system consists of both CD4 T-helper cells and CD8 cytotoxic cells, and both play a pivotal role in the control of HCMV infection. Initial T-cell recovery following allogeneic transplantation is of phenotypically mature antigen primed memory CD45RA+ T cells, with a limited diversity based on analysis of the T-cell receptor repertoire, particularly following T-cell depleted transplants (Roux et al., 1996). By measuring T-cell receptor rearrangement excision circles (TRECs) it can be shown that early thymic dependent naïve T cell recovery becomes active between 6 and 12 months post transplant, while increasing age, chronic GVHD and T-cell depletion predicted delayed thymic recovery (Fallen et al., 2003). This recovery of thymic function is characterised by the appearance of CD4+ CD45RA+ RO- T cells which are of donor origin (Roux et al., 2000). The absolute CD4 levels remains very low for the first few months post transplant, gradually rising to a mean of 300 cells/μL at

one year, but even five years post transplant does not achieve normal levels (Storek et al., 1995).

Absolute CD8+ T cell counts also remain low in the first three months following allogeneic transplantion, usually less than 200 cells/μL, but achieve normal or supranormal levels by one year (Storek et al., 1995). Similar to CD4+ T cells, the phenotype is that of an antigen primed cell with high expression of CD11a, CD11b, CD29, CD57, and HLA-DR but low expression of CD28, CD45RA, and CD62L compared to normal individuals. The increased expression of CD57, CD11b, as well as the absence of CD28 expression suggests much of the numerical increase is functionally anergic (Dolstra et al., 1996). Early studies of antigen specific responses including to HCMV as well as HLA specific cytotoxicity assays suggest a defect early post allogeneic stem cell transplant, but as these experiments were done on total mononuclear preps, they were not quantitative (Quinnan, Jr. et al., 1981; Reusser et al., 1991; Riddell et al., 1992; Walter et al., 1995). Later studies using limiting dilution assays suggested the defect in fact to be quantitative (Lucas et al., 1996). It is noteworthy that HCMV seropositive recipients with a seropositive donor have a greater HLA Class 1 restricted HCMV specific response than patients with seronegative donors (Li et al., 1994). Walter showed that in allogeneic transplant recipients the HCMV specific CD8 response is impaired in the absence of a CD4 response (Walter et al., 1995) .

1.12 Clinical Features of Human Cytomegalovirus Infection

1.12.1 Infection in the Immunocompetent

HCMV infection in the normal healthy individual is usually asymptomatic but may present as an 'infectious mononucleosis' like syndrome although heterophile antibody negative (Zanghellini et al., 1999). Clinical features of HCMV infection include fever in the majority of cases, as well as lymphadenopathy, pharyngitis and splenomegaly, while a rash is present in 30 percent of infected individuals. Over 90 percent have a mild elevation of liver transaminases, and an elevation of the lymphocyte count with atypical lymphocytes on blood film occuring in the majority of individuals.

In 1960 the so called 'post perfusion syndrome' was described, consisting of fever, splenomegaly, atypical lymphocytosis and raised HCMV antibody titres 3 to 4 weeks following open heart surgery, and was attributed to the receipt of blood products infected with HCMV (Lang et al., 1968). This syndrome is now prevented by the leukodepletion of blood products (Pamphilon et al., 1999).

Despite the very high prevalence of latent HCMV infection in the general population, relatively few HCMV disease associations in otherwise healthy individuals have been made. HCMV DNA as well as latent HCMV virus can be found in the arterial walls of patients with severe atherosclerosis (Hendrix et al., 1990; Melnick et al., 1993). Furthermore, coronary restenosis has been associated with HCMV infection (Speir et

al., 1994). Atherosclerosis is an inflammatory process (Ross, 1999) and it has been suggested that HCMV infection plays a crucial role in mediating atherosclerosis through the induction of adhesion molecules and growth factors (Westphal et al., 2006). There is also some evidence that early onset pre-eclampsia, a disease caused by arteriole insufficiency may also be associated with HCMV infection (Carreiras et al., 2002; von Dadelszen et al., 2003).

Recently it has become recognized that HCMV contributes to age related immunosenescence. Among the elderly, the immune response to HCMV becomes increasingly focused towards single dominant epitopes (Khan et al., 2002b). An HCMV specific immunophenotype of CD8+ CD57+ T cells with loss of CD45RA and CD28 expression occurs (Ouyang et al., 2003). Reversal of the CD4/CD8 ratio and expansion of CD8+ T cell population was associated with HCMV seropositivity and predictive of an increased risk of death over the subsequent one to two years (Olsson et al., 2000; Wikby et al., 2002). Furthermore, the ability to respond to new antigenic challenges is impaired, as demonstrated by the reduced immune response to influenza vaccination in HCMV seropositive elderly people (Trzonkowski et al., 2003).

1.12.2 Infection in the Immunosuppressed Host

In contrast to the situation in healthy individuals, HCMV infection in patients with impaired immune function, particularly T-cell immunity, can result in significant morbidity and mortality. Immunodeficiency as a result of immaturity (such as the fetus or very premature neonate), acquired immunodeficiency due to infection with the human immunodeficiency

virus (HIV), congenital immunodeficiency states (Raeiszadeh et al., 2006), iatrogenic immunosuppression in the context of solid organ transplantation and bone marrow transplantation, and monoclonal antibody therapy for the treatment of lymphoproliferative disorders (Bowen et al., 1997) are all conditions that render patients susceptible to HCMV disease. Infection may occur because of **primary infection** (such as transplant of an HCMV seropositive graft into an HCMV seronegative recipient), **reactivation** of primary infection, or **reinfection** with a new strain in an HCMV seropositive patients with primary infection in the past. The disease manifestation of HCMV differs according to the underlying condition, and this may reflects differences in the nature of the immunodeficiency in these patients.

Congenital infection

Congenital HCMV infection occurs in 1 percent of all live births, and 18 percent of congenitally infected infants have clinical manifestation of HCMV disease such as microcephaly, mental retardation, blindness, deafness as well as non-central nervous system manifestations such as thrombocytopaenia, hepatitis, pneumonitis and myocarditis (Fowler et al., 1992). However between 7 and 25 percent of congenitally infected asymptomatic infants at birth present with sensorineural hearing loss at up to four years of age (Iwasaki et al., 2007; Fowler et al., 1997), and congenital HCMV infection is the most common cause of childhood deafness. The greatest risk of congenital HCMV infection is in the infants of seronegative mothers experiencing primary infection during pregnancy (Fowler et al., 1992), although there is evidence that re-infection with a

new strain in a seropositive mother also confers an increased risk (Boppana et al., 2001). HCMV is the most common cause of congenital infection of the newborn, and for this reason, there is a strong argument for the development of an effective vaccination.

Solid organ transplantation

HCMV infection following a kidney transplantation typically presents 1 to 4 months post allografting (Rubin, 1990), and may manifest as anything from a mild viral illness with fever, rash and leukopaenia to more severe organ dysfunction including pneumonitis, hepatitis and gastro-enteritis. Kidney transplantation between an HCMV seropositivity donor and a seronegative recipient (D+/R+) carries the highest incidence (40-73 percent) of HCMV disease (Lowance et al., 1999), and is due to primary infection from the graft. HCMV infection following liver transplant is associated with hepatitis (Rubin, 1997). HCMV following cardiac transplantation has been associated with accelerated atherosclerosis and transplant vasculopathy (Fateh-Moghadam et al., 2003), while HCMV infection significantly increases the risk of obliterative bronchiolitis in lung or heart-lung transplants (Cerrina et al., 1992). As well as the direct effects of HCMV infection, there are indirect consequences on solid organ transplantation, including an increased risk of graft rejection and secondary infections. HCMV disease (Toupance et al., 2000) and according to some authors also HCMV infection (Sageda et al., 2002) is an independent risk factors for acute rejection, as well as being implicated as a cause of chronic allograft nephropathy (Yates and Nicholson, 2006).

HIV

Prior to the advent of HAART therapy for HIV patients, between 10 and 30 percent of patients with advanced HIV infection developed HCMV disease per year (Shepp et al., 1996). The main risk factor predicting HCMV infection is the CD4 count, with patients with a CD4 count less then 100/μL being at the greatest risk. Unlike solid organ transplant recipients, the major disease manifestation of HCMV infection in HIV patients is retinitis, while encephalopathy, polyradiculopathy, gastroenteritis, and hepatitis are also common. Since the introduction of HAART, the incidence of HCMV retinitis has decreased dramatically (Deayton et al., 2000), although even in the era of HAART, detection of HCMV viraemia remains an independent risk factor for death (Deayton et al., 2004).

1.13 HCMV Infection in Allogeneic Stem Cell Transplantation

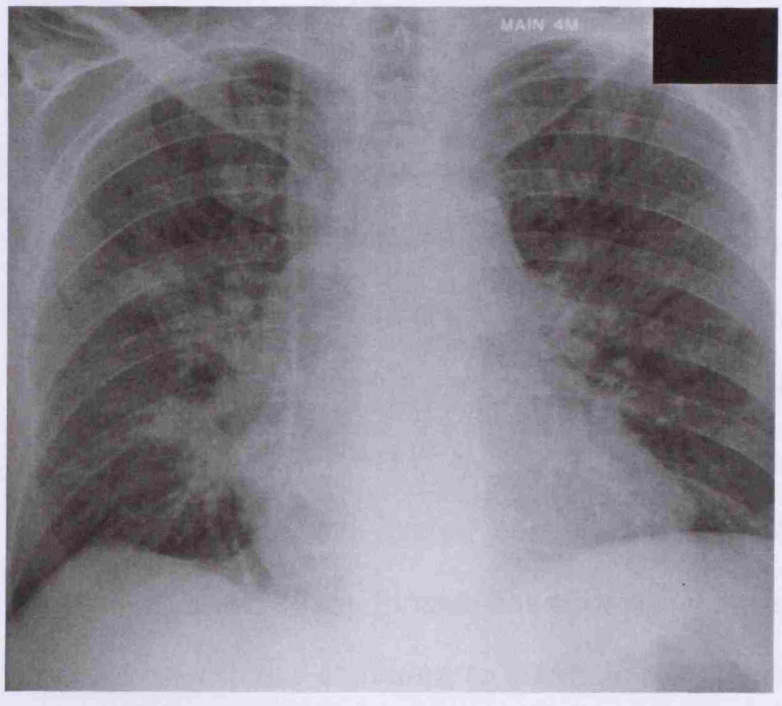
Prior to the advent of sensitive surveillance methods and effective antiviral therapy for human cytomegalovirus (HCMV), interstitial pneumonitis due to HCMV occurred in 10-20 percent of stem cell transplant recipients and untreated has a mortality of greater then 80 percent (Meyers et al., 1986). Despite therapy with ganciclovir and intravenous immunoglobulin, mortality of established HCMV disease remains high (Emanuel et al., 1988; Ljungman et al., 1992; Reed et al., 1988). The main source of infection is reactivation of latent infection in the recipient (Winston et al., 1985), although in HCMV seronegative

recipients, unfiltered blood products or unmanipulated marrow from HCMV seropositive donors can also give rise to a primary infection (Meyers et al., 1986). By contrast HCMV infection is infrequent following autologous stem cell transplant, with HCMV pneumonia being a rare occurrence (Konoplev et al., 2001).

1.13.1 Disease Manifestations

The most common disease manifestation of HCMV disease following stem cell transplantation is HCMV pneumonitis, and is initially characterised by fever, cough, and hypoxia with diffuse interstitial changes on X-ray before progressing to respiratory failure (Ljungman, 1995; Salomon and Perlman, 1999). Figure 1.3 is an example of the chest X-ray changes of HCMV pneumonitis. It is likely that the pathogenesis of HCMV pneumonitis in the immunocompromised host has an immunological basis (Riddell, 1995; Grundy et al., 1987). HCMV hepatitis is characterised by hepatic dysfunction while liver biopsy may show typical viral intranuclear inclusions. Gut involvement by HCMV includes colitis, which typically presents with diarrhoea and abdominal pain but may also cause life-threatening haemorrhage, as well as gastritis and oesophagitis. HCMV retinitis is a late complication of HCMV infection in allogeneic transplant recipients but appears to be increasing in frequency (Crippa et al., 2001; Larsson et al., 2002; Xhaard et al., 2007). Formal definitions of HCMV disease were first defined by the First International CMV Workshop in 1993 and have subsequently been updated. (Ljungman et al., 2002b).

Figure 1-3 Chest X-ray changes of HCMV pneumonitis in an allogeneic stem cell transplant recipient.



1.13.2 Risk Factors

Previously identified risk factors for HCMV disease include HCMV seropositivity, GVHD, total body irradiation, and asymptomatic HCMV infection (Goodrich et al., 1991; Meyers et al., 1986; Meyers et al., 1990; Wingard et al., 1988), while positive donor HCMV serology may be protective (Grob et al., 1987). Acute GVHD is associated with HCMV infection post BMT (Miller et al., 1986), and there is good evidence that the alloimmune response can stimulate HCMV reactivation from the latent state (Soderberg-Naucler et al., 1997; Winston et al., 1985). T-cell depletion has also been identified as a significant risk factor for HCMV infection and disease (Couriel et al., 1996; Hertenstein et al., 1995), as has the use of unrelated donors (Takenaka et al., 1997). Following reduced intensity conditioning regimens incorporating ATG or alemtuzumab, HCMV infection rates are increased (Chakrabarti et al.,

2002a; Mohty et al., 2000), although the Seattle group using a low dose TBI based reduced intensity conditioning regimen have reported no differences in the rate of HCMV infection at 12 months (Junghanss et al., 2002).

Blood based dissemination of the virus is an important precursor of later disease, with nearly all allogeneic stem cell transplant recipients with HCMV disease experiencing a preceding viraemia (Schmidt et al., 1991; Enright et al., 1993; Meyers et al., 1990). HCMV antigeneamia has been correlated with transplant related mortality due to HCMV disease (Bacigalupo et al., 1995). Einsele has shown that patients requiring greater than 4 weeks of therapy to eliminate HCMV infection during pre-emptive therapy are at an increased risk of late HCMV disease, while the presence of HCMV viraemia prior to transplant is associated with a very high risk of disease (Einsele et al., 2000).

Using a quantitative competitive PCR technique, it has been demonstrated that the major risk factor predicting HCMV disease is the peak viral load at the time of HCMV infection (Gor et al., 1998). The presence of HCMV disease prior to transplantation is associated with a very high risk of early HCMV disease and death after transplantation (Fries et al., 2005).

1.13.3 Indirect Effects

HCMV infection is a recognized risk factor for aspergillus infection in lung transplant recipients (Husni et al., 1998), and the high risk of death due to bacterial and fungal infection among HCMV seronegative recipients of stem cell transplants from seropositive donors provides further evidence

for indirect effects of primary HCMV infection (Nichols et al., 2002). In vitro, HCMV infection is myelosuppressive, both by directly infecting myeloid progenitors and indirectly through a cytokine mediated mechanism (Torok-Storb et al., 1992), and it is likely that a similar mechanism occurs in vivo.

1.13.4 Impact of HCMV Serology on Transplant Mortality

A Dutch study reported that in matched unrelated donor transplants but not related donor transplants, recipient HCMV seropositivity adversely affected overall survival and transplant related mortality (Meijer et al., 2002).

A large EBMT metafile analysis showed that for HLA identical sibling allografts, HCMV serostatus of the donor had no influence on survival of HCMV seropositive recipients, but for unrelated donors there was a reduced transplant related mortality and a significant survival advantage if HCMV seropositive donors were used (Ljungman et al., 2003a). Another recent study in reduced intensity conditioned transplants suggested that HCMV donor seropositivity reduced the incidence of leukaemia relapse but this did not translate into improved survival due to increased treatment related mortality (Nachbaur et al., 2006). In the setting of T-cell depleted transplants for CML, Craddock has reported that HCMV donor seropositivity is associated with adverse transplant outcomes (Craddock et al., 2001).

1.14 Laboratory Diagnosis and Monitoring

1.14.1 Serology

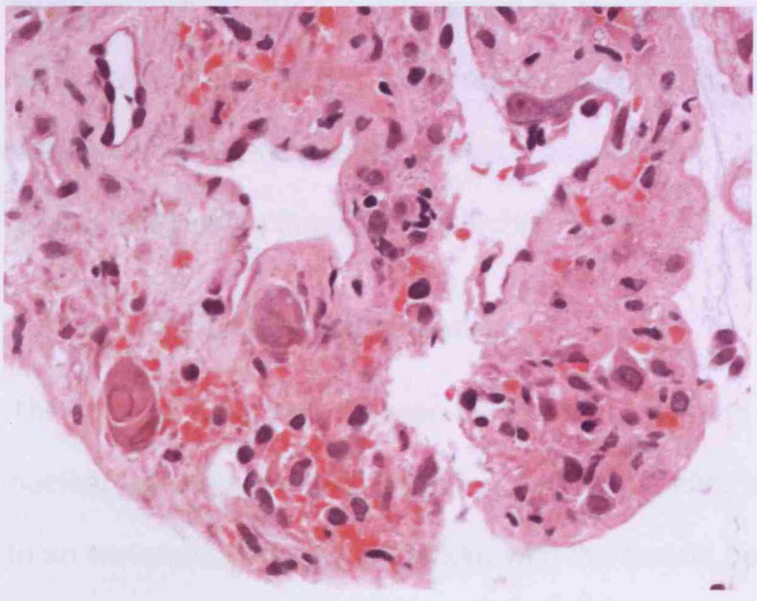
HCMV IgG seropositivity is indicative of past infection, and it is essential to establish the serostatus of both the donor and recipient prior to transplant. HCMV IgM positivity in the non-transplant setting is indicative of recent infection. The urea denaturation test removes low avidity antibodies formed early following infection, and can be used to document recent infection in IgG seropositive patients, such as pregnant woman who may be at risk of congenital HCMV infection (Blackburn et al., 1991). Following allogeneic stem cell transplant, patients with an HCMV seropositive donor typically show a secondary antibody response with rising levels of high avidity antibodies, while patients with seronegative donors show a primary pattern of antibody response, charactertised by low avidity maturing to high avidity antibodies, at approximately 250 days post transplant (Lutz et al., 1996). HCMV serology cannot be used to diagnose active infection in the allogeneic transplant recipeint.

1.14.2 Tissue Diagnosis

The characteristic histopathological feature of HCMV disease is the intranuclear 'owl eye inclusion body', which is highly specific and correlates with the HCMV viral load, although significantly less sensitive then molecular techniques to detect the virus in the same organ (Mattes et al., 2000). Figure 1-4 shows a haematoxylin & eosin (H&E) stained slide of a colonic biopsy from a patient developing HCMV colitis 64 days following a reduced intensity unrelated donor allogeneic transplant.

Immunochemical and DNA hybridisation techniques may also be used for histological diagnosis of HCMV disease.

Figure 1-4 H&E stained slide of a colon biopsy of an allogeneic stem cell transplant patient with HCMV colitis showing typical viral inclusion bodies (personal collection).



1.14.3 Cell Culture Techniques

Cell culture

Conventional cell culture techniques to detect HCMV rely on the detection of a cytopathic effect (CPE) in human foreskin fibroblast cultures, but although cell culture has 100% specificity, the test requires up to six weeks incubation. Detection of early antigen fluorescent foci (DEAFF) using monoclonal antibodies, combined with the shell vial culture system which uses centrifugation to inoculate a monolayer of fibroblasts, can shorten the procedure to 24 hours (Griffiths et al., 1984). However culture techniques are limited by low sensitivity and rapid loss of viability of stored samples (Boeckh and Boivin, 1998). Viral quantification can be calculated from serial dilutions of the sample. There are three methods of

quantifying HCMV by culture. The plaque assay uses serial dilutions of sample onto fibroblast monolayers, and looks for plaque formation (virus spreads from cell to cell). The infective dose to cause 50% infection of tissue culture ($TCID_{50}$) is determined by serial dilutions of a sample, which are cultured onto human foreskin fibroblasts and observed for six weeks. The third technique is direct measurement of the number of infective foci using the shell vial technique.

1.14.4 pp65 Antigenaemia

The antigenaemia test is based on the direct staining of polymorpho-nuclear leukocytes (PMN) by monoclonal antibodies to pp65 conjugated to an immunofluorescence marker, with the results being expressed as the number of antigen positive cells relative to the number of cells used to prepare the slide. The original description of the method used 150000 cells per slide (van der Bij et al., 1988), but to reduce error when low level viraemia is present it is recommended to perform the test on 2 to 3 slides (Boeckh and Boivin, 1998). Compared to cell culture, antigenaemia has the same specificity but greater sensitivity and can be performed in less than three hours (Erice et al., 1995; Ho et al., 1998). The major limitation of the antigenaemia assay is the requirement for sufficient number of PMNs, a criteria that may not be fulfilled early post stem cell transplantation, a time when the risk of HCMV infection is greatest (Boeckh et al., 1992).

1.14.5 Polymerase Chain Techniques

Qualitative PCR

Qualitative HCMV DNA can be used to detect HCMV viraemia transplant recipients (Jiwa et al., 1989). Symptomatic HCMV infection in allogeneic transplant recipients as well as resolution of infection following ganciclovir therapy correlates with the detection of HCMV by PCR in blood and urine (Einsele et al., 1991). PCR is significantly more sensitive then cell culture, and HCMV can be detected in blood by PCR a mean of five days prior to onset of disease, compared to 13 days after the onset of disease for conventional cell culture techniques (Kidd et al., 1993). Although HCMV is a cellular virus, it can also be detected in the cell free component of blood (Gerna et al., 1992; Grefte et al., 1994; Revello et al., 1992). In allogeneic transplant patients, PCR of whole blood was shown to be more sensitive in one study (Nolte et al., 1995) while most studies find either no difference (Hebart et al., 1996) or whole blood to be less sensitive (Boeckh et al., 1997). A recent UK based study of renal and stem cell transplant patients demonstrated that HCMV monitoring by PCR is more sensitive and specific than the pp65 antigenaemia test, and significantly less expensive (Szczepura et al., 2006).

Quantitative

Quantitation of DNA by competitive PCR can be performed by adding a PCR competitor with identical primer sites but a restriction enzyme digestion site engineered into it, so the amplification of the differentiation products can be run out on a gel. This has been developed for HCMV

using a homologous gB competitor with the exception of a unique restriction site (Fox et al., 1992). Although viral load monitoring can also be used for monitoring response to therapy (Gerna et al., 1995), the technique is cumbersome, labour and time intensive and is not suitable for routine use, batch processing.

1.15 Antiviral Therapy

The first drugs to show significant antiviral activity against the herpesviruses with tolerable side effect profiles were the nucleoside analogues first synthesised in the 1970s, although ganciclovir was not developed until 1982 (Verheyden, 1988). Other drugs with activity against HCMV include foscarnet, cidofovir and the antisense nucleotide, formivirsen. The biochemical structure of drugs with activity against HCMV is shown in Figure 1-5.

1.15.1 Aciclovir

Aciclovir or 9-(2-hydroxethoxymethyl) guanine is a guanosine nucleoside analogue, which competes with dGTP for the viral DNA polymerase, and causes termination of DNA synthesis by preventing the formation of phosphodiester bonds. Before the drug is active, it must undergo phosphorylation to the monophosphate form by the viral enzyme, thymidine kinase, followed by further phosphorylation by cellular enzymes, which convert it to the active aciclovir triphosphate form. Toxicity to non-infected cells is minimal as the enzyme for the first phosphorylation is not present in uninfected cells, although aciclovir can cause renal impairment and rarely neurological dysfunction. Its spectrum

of activity includes HSV 1 and 2, and VZV. It was initially thought that aciclovir was not active against HCMV, but the HCMV phosphotransferase encoded by UL97 is capable of phosphorylating aciclovir (Talarico et al., 1999), and aciclovir has partial activity against HCMV (Tyms et al., 1981).

As the oral bioavailability of aciclovir is poor, the 1-valyl ester of aciclovir, valaciclovir has been formulated. Hydrolysis by a valine esterase occurs in the small intestine and results in a substantially higher oral bioavailability of approximately 60 percent, and hence higher serum levels. Valaciclovir has been evaluated as prophylaxis for HCMV infection in both solid organ (Lowance et al., 1999; Fiddian et al., 2002) and stem cell transplant patients (Ljungman et al., 2002a) and shown to be effective. In the high risk setting of alemtuzumab conditioned stem cell transplant, the combination of pre-transplant ganciclovir and post-transplant oral valaciclovir reduced the incidence of HCMV infection from 53 percent to 29 percent (Kline et al., 2006). The higher dose of serum levels of aciclovir may result in formation of urine crystals, so good urine output needs to be maintained.

1.15.2 Ganciclovir

Ganciclovir or 9-(1,3-dihydroxy-2-propoxy) methylguanine is also a guanosine analogue, differing from aciclovir by the presence of a hydroxymethyl group. Its spectrum of activity includes HSV and HCMV, and is licensed for use in HCMV. Initial phosphorylation by the HCMV specific phophotransferase UL97 (Littler et al., 1992) followed by further phosphorylation to ganciclovir triphosphate by cellular kinases is required

for activity.The oral bioavailability of ganciclovir is poor, estimated at approximately 7 percent in stem cell transplant patients (Boeckh et al., 1998b). The plasma half-life is 3-4 hours, and clearance is by renal excretion, although in the presence of renal impairment, the half-life is significantly prolonged. Major side effects include neutropaenia, which is more severe in stem cell transplant recipients (Reed et al., 1990), thrombocytopaenia and nephrotoxicity.

Low level resistance to ganciclovir may occur due to substitution or deletion mutation of the catalytic domain of UL97 (Baldanti et al., 1995; Chou et al., 1995b; Chou et al., 1995a; Sullivan et al., 1993). High level resistance occurs as a result of mutation in the DNA polymerase, UL54 (Smith et al., 1997), which also results in cross resistance to foscarnet. Ganciclovir resistance is uncommon in the allogeneic stem cell transplant setting (Reusser et al., 1996), and generally only occurs following prolonged therapy (Erice et al., 1998).

Valganciclovir, the L-valyl ester of ganciclovir, like valaciclovir, undergoes hydrolysis by a valine esterase in the intestine, with a markedly improved bioavailability. A number of small trials have evaluated valganciclovir in allogeneic transplant patients (Ayala et al., 2006; van der Heiden et al., 2006). Valganciclovir at a dose of 900mg twice daily has an equivalent efficacy and safety profile to intravenous ganciclvoir at a dose of 5mg/kg twice daily in patients with gut GVHD and HCMV infection, although oral valganciclovir results in higher blood levels of ganciclovir, and oral bioavailability is estimated to be 75 percent (Einsele et al., 2006).

1.15.3 Foscarnet

Foscarnet or trisodium phophonoformate is not a nucleoside analogue but a pyrophosphate analog. It has activity against all herpesviruses as well as Hepatitis B and HIV. It is a non-competitive inhibitor of viral DNA polymerase. Resistance can occur because of mutations in the viral DNA polymerase UL54, which also result in cross-resistance to ganciclovir as discussed above. It is renally excreted, and the major toxicity is nephrotoxicity, which can be limited by saline prehydration (Deray et al., 1989). Metabolic disturbances are common, including hypo or hyperkalaemia, hypomagnesaemia, hypo or hyperphosphataemia, and calcium abnormalities. Other side effects include nausea and vomiting as well as neurologic toxicities including tremor, altered sensation, and seizures. Foscarnet at a dose of 60mg/kg twice daily is effective as monotherapy for HCMV infection in bone marrow transplantation (Ljungman et al., 1996b), and has equal efficacy to ganciclovir 5mg/kg twice daily with a lower incidence of neutropaenia (Reusser et al., 2002).

1.15.4 Cidofovir

Cidofovir or (S)-1-(3-hydroxy-2-phosphoryl methoxypropyl) cytosine, is a monophosphate that is converted intracellularly to the diphosphorylated form by the actions of cellular kinases. It acts as an analogue of the nucleoside dCTP, to inhibit viral DNA polymerase, causing premature DNA chain termination. The main indication for cidofovir is the treatment of HCMV infection but it has also been used to treat adenovirus infections following allogeneic transplants (Feuchtinger et al., 2007; Ljungman et al.,

2003b). Although the serum half-life is only 2.6 hours, the intracellular half-life is greater then 48 hours, allowing intermittent dosing (Cundy et al., 1996). Its use is complicated by a high incidence of nephrotoxicity due to tubular damage (Polis et al., 1995), with other common side effects including fever, nausea and rash. Probenecid administration pre and post infusion reduces renal clearance and allows weekly or biweekly dosing, while saline prehydration reduces renal toxicity. Viral resistance can occur due to mutations in the HCMV DNA polymerase gene, with cross resistance to ganciclovir but not foscarnet (Emery, 1998). Cidofovir is currently used as second line therapy for HCMV infection in allogeneic stem cell transplant recipients (Ljungman et al., 2001a).

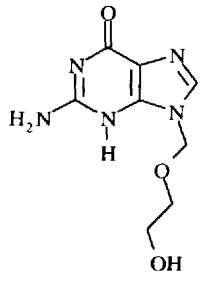
1.15.5 Formivirsen

Formivirsen is an antisense oligonucleotide, which binds to the mRNA of IE2. It is licensed for intra- vitreal administration for the treatment of HCMV retinitis (de Clercq E., 2003).

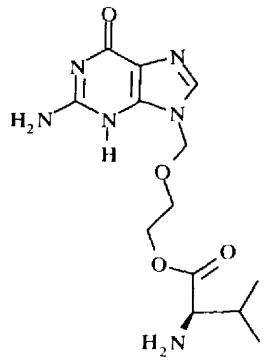
1.15.6 Combination Therapy

While the main toxicity of ganciclovir is marrow suppression and for foscarnet is renal and metabolic, combining both drugs at 50 percent of the dose used for monotherapy results in reduced toxicity. This regimen has been studied in both solid and allogeneic stem cell transplant recipients and has similar efficacy to ganciclovir therapy (Mattes et al., 2004), although there is no evidence to suggest a synergistic effect.

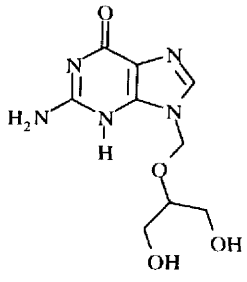
Figure 1-5 Chemical structure of commonly used drugs for the treatment of human cytomegalovirus infections.



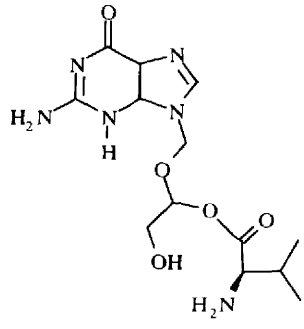
Aciclovir



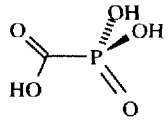
Valaciclovir



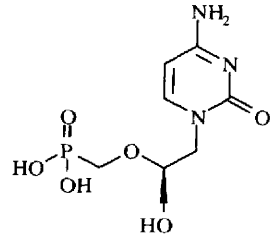
Ganciclovir



Valganciclovir



Foscarnet



Cidofovir

1.16 Strategies for the Prevention and Treatment of HCMV Disease in Allogeneic Stem Cell Transplantation

1.16.1 Prevention

HCMV seronegative allotransplant recipients receiving HCMV unselected blood products have a risk of transfusion-associated infection of between 28 and 57 percent (Bowden et al., 1986; Miller et al., 1991). In HCMV seronegative recipients, HCMV infection can be prevented by the use of a negative donor if available, and by the use of HCMV seronegative blood products or leukocyte depleted blood products (Bowden et al., 1991; Verdonck et al., 1987). A study comparing the use of filters that reduce leukocytes in blood by three logs with seronegative blood products show that both approaches are equally efficacious in preventing HCMV infection, although a small but statistically significant greater number of patients receiving filtered blood experienced HCMV disease (Bowden et al., 1995).

1.16.2 Treatment of established disease

Once HCMV disease is established, mortality is very high. Although outcomes have improved with the administration of ganciclovir and intravenous immunoglobulin, survival rates remains poor with only 31 percent of patients with HCMV pneumonitis surviving (Emanuel et al., 1988; Ljungman et al., 1992; Reed et al., 1988; Schmidt et al., 1988). Use of intravenous ganciclovir for two weeks for the treatment of cytomegalovirus gastroenteritis was not associated with clinical improvement (Reed et al., 1990). Because of the very high mortality of

HCMV infection in the stem cell patient, prevention of clinical disease should be the goal of any treatment strategies.

1.16.3 Intravenous Immunoglobulin

Although early reports support the use of intravenous immunoglobulin in combination with ganciclovir for the treatment of HCMV disease, there is little evidence for a beneficial effect in the prevention of HCMV infection in allogeneic stem cell transplantation (Sokos et al., 2002). There is some evidence for a role for both intravenous immunoglobulin or hyperimmune HCMV immunoglobulin in combination with ganciclovir, but not alone in preventing HCMV disease in the solid organ setting, particularly following heart and lung transplant (Bonaros et al., 2004; Garcia-Gallo et al., 2005; Kocher et al., 2003; Kruger et al., 2003; Weill et al., 2003). High titre HCMV immune globulin can ameliorate HCMV disease in solid organ transplant (Snydman et al., 1987). However, a large trial using HCMV hyperimmune immunoglobulin in allogeneic stem cell transplant recipients provided no benefit (Ringden et al., 1987).

1.16.4 Prophylactic Aciclovir

Aciclovir has been show to reduce HCMV infection and improve survival in some (Meyers et al., 1988; Prentice et al., 1994) but not all clinical trials (Boeckh et al., 1998a). The initial study by Prentice showed that use of high dose intravenous followed by oral aciclovir in stem cell recipients at risk of infection reduced the frequency and time to infection, but could not show a reduction in frequency of disease, although a follow-up study showed a significant survival advantage for high dose aciclovir (Prentice

et al., 1997). A recent study incorporating a strategy of pre-transplant prophylactic ganciclovir, post-transplant acyclovir and pre-emptive ganciclovir for HCMV infection has proved to be very effective at preventing HCMV disease (Verma et al., 2003). The aciclovir prodrug, valaciclovir, with its higher oral bioavailability, has been used to achieve higher serum levels of aciclovir, and has been evaluated in a large trial in the renal transplant setting (Lowance et al., 1999). Studies have also been performed in the stem cell setting (Mori et al., 2006; Winston et al., 2003; Ljungman et al., 2002a; Vusirikala et al., 2001), and show a reduced incidence of HCMV infection when compared to aciclovir, although there was no difference in HCMV disease.

1.16.5 Prophylactic Ganciclovir

Universal prophylactic administration of ganciclovir for all patients at risk of HCMV infection by virtue of a positive donor and or recipient serology reduces the incidence of HCMV infection and disease in both solid organ transplant and allogeneic stem cell transplantation. In the stem cell transplant setting, the dose of ganciclovir used is lower (5mg/kg daily) then the therapeutic dose, although a lower dose of 5mg/kg three times per week has also used. Although effective at reducing HCMV disease among stem cell patients, there is no decrease in overall mortality (Goodrich et al., 1993; Boeckh et al., 1996; Winston et al., 1993), most likely due to an increased frequency of bacterial and fungal infection secondary to neutropaenia (Salzberger et al., 1997). Thus, many patients receive ganciclovir unnecessarily and are exposed to the myeolosuppressive side effects. The recovery of HCMV specific immunity

is also delayed when prophylactic ganciclovir is used (Li et al., 1994), and this increases the risk of late HCMV infection.

1.16.6 Surveillance and Pre-emptive Therapy

Pre-emptive therapy utilises highly sensitive methods to identify patients at risk for disease from HCMV prior to the onset of disease, targeting antiviral therapy only to those patients requiring it. Initial attempts at pre-emptive therapy were based on surveillance cultures taken at set times following transplantation (Goodrich et al., 1991) or surveillance by BAL (Forman and Zaia, 1994; Schmidt et al., 1991). Schmidt undertook a trial of prophylactic ganciclovir, initiated when a surveillance BAL was culture positive for HCMV, and showed a significant reduction in HCMV disease, and no deaths due to HCMV interstitial pneumonitis when compared to patients with a positive BAL not given ganciclovir. This was an effective approach at identifying patients at risk of disease, but often disease was already present by the time cultures results were available. This lead to the development of more rapid and sensitive techniques to detect viraemia. The antigenaemia test has been used extensively for surveillance as part of a pre-emptive therapy program (Bacigalupo et al., 1995; Boeckh et al., 1992), and compares favourably with HCMV culture techniques (Nicholson et al., 1997). Although rapid, with a potential turnaround time of two hours, it is limited by being dependent on a sufficient neutrophil count. This has lead to many transplant units adopting qualitative PCR of peripheral blood for surveillance strategies (Einsele et al., 1995; Ljungman et al., 1996a). In comparison with antigenaemia, the PCR technique is more sensitive and not dependent

on the leukocyte count (Boeckh et al., 1997; Hebart et al., 1996; Vlieger et al., 1992). Few studies have directly compared a pre-emptive strategy with a prophylactic strategy as part of a randomized trial. Boeckh found a greater frequency of HCMV disease with a pre-emptive approach, but greater fungal infection and late disease with universal ganciclovir prophylaxis, and no difference in survival, although in this trial, ganciclovir was only initiated at high level antigenaemia, and not on the basis of a positive PCR (Boeckh et al., 1996). In the United States, the majority of allogeneic stem cell transplant programs operate an exclusively pre-emptive program (Avery et al., 2000). Ganciclovir doses used for pre-emptive therapy have generally been 5mg/kg twice daily, but a trial evaluating low dose pre-emptive ganciclovir at 5mg/kg per day in patients receiving peripheral blood stem cell transplants has also been evaluated (Vij et al., 2003). Although the authors argue that this dose is effective, they do report a 9 percent incidence of HCMV disease in patients treated in this way. Pre-emptive therapy can safely be discontinued once a patient becomes PCR negative (Einsele et al., 1995).

1.17 New Approaches for the Prevention or Treatment of HCMV Infections in Allogeneic Stem Cell Transplant Recipients

1.17.1 Immunotherapy

The infusion of HCMV specific donor cells enhances the HCMV specific immunity of the recipient and provides protection against recurrence of infection and HCMV disease. Walter et al described a study of the

infusion of HCMV specific CD8+ cytotoxic T cell clones, generated by stimulating donor PBMCs with HCMV infected donor derived fibroblasts (Walter et al., 1995). They demonstrated by T-cell receptor rearrangement studies that these clones were not present in the recipient prior to infusion and persisted for at least 12 weeks. A major shortcoming in this study is that a live virus is required to generate the HCMV specific cell lines. Peggs et al described an approach of generating both CD8+ and CD4+ HCMV specific T-cell lines by stimulating peripheral blood lymphocytes from HCMV seropositive donors with autologus monocyte derived dendritic cells pulsed with HCMV viral lysate (Peggs et al., 2001). Einsele's group used donor derived PBMC's, stimulated directly with HCMV viral lysate to generate HCMV specific cell lines (Einsele et al., 2002). However, the generation of HCMV specific cell lines is time consuming. Furthermore, although these studies were effective in controlling HCMV infection, the use of viral lysate carries a risk of viral contamination of the therapeutic product. Thus, this approach cannot confirm to good manufacturing practices. An alternative strategy is to select HCMV specific CD8+ T cells using Class I restricted HCMV specific tetramers combined with immunomagnetic beads (Cobbold et al., 2005). The process is rapid, and despite the relatively low cell dose infused appears to be effective. This technique is limited to known Class I HCMV epitopes for which tetramers are available, and does not provide a source of HCMV specific CD4+ T-cells. An immunomagnetic interferon gamma capture system can also be used to isolate HCMV specific T-cells following stimulation of donor blood with HCMV specific Class I and II

peptides (Rauser et al., 2004). These cells can then be expanded, generating large numbers of HCMV reactive but allodepleted cells in a technique that conforms to good manufacturing practice. More recently, workers at the Sloan-Kettering Cancer Centre evaluated the use of overlapping peptides for the immunodominant HCMV protein, pp65, to generate T-cell lines, an approach which is not restricted to known HCMV specific class I and II epitopes (Trivedi et al., 2005).

1.17.2 HCMV vaccine

Although significant progress in immunotherapy for HCMV has been made, it is still a requirement for the donor to be seropositive for HCMV. Strategies for HCMV vaccination include protein subunit vaccines, live attenuated vaccines, viral vector vaccines, peptide vaccines and DNA vaccines, but no licensed vaccine for HCMV is available yet (Schleiss and Heineman, 2005). The Towne vaccine, a live attenuated virus (a highly passage strain of HCMV) has been used for vaccination in renal transplant recipients (Quinnan, Jr. et al., 1984; Plotkin et al., 1976). Although it did not reduce the frequency of HCMV infection in seronegative recipients of seropositive grafts, the incidence of HCMV disease, in paticularly severe disease was reduced, similar to a level seen in seropositive renal recipients (Plotkin et al., 1984; Plotkin et al., 1991). The canary pox-CMV gB recombinant virus has been successfully used to generate a humoral gB specific response to HCMV (Adler et al., 1999). In order to generate a CTL response, a canary pox CMV pp65 recombinant virus was used successfully (Berencsi et al., 2001).

A subunit vaccine using gB and a novel adjuvant, MF59, has also had some success in generating humoral response (Pass et al., 1999). Novel approaches to HCMV vaccination includes the use of DNA vaccines and peptide vaccines, which have been evaluated in animal models (Gallez-Hawkins et al., 2004; Gopal et al., 2005; Schleiss et al., 2006). Universal HCMV vaccination to prevent congenital HCMV infection has been assigned the highest priority by the United States of Ameirica Institute of Medicine (Arvin et al., 2004), and would also have the additional benefit of eventually eliminating the problem of HCMV infection in solid organ and stem cell transplants.

1.18 Aims of the Thesis

1. To identify the risk factors for HCMV infection in allogeneic stem cell transplant recipients receiving high dose aciclovir prophylaxis, HCMV surveillance by qualitative PCR, and pre-emptive therapy for infection.
2. To evaluate the technology of the TaqMan method for real time PCR viral load monitoring, to assess the predictive value of the viral load when using a strategy of pre-emptive therapy to prevent HCMV disease, and to assess the utility of real time PCR in predicting the likely response to HCMV antiviral therapy in allogeneic stem cell transplant recipients.
3. To evaluate the role of HCMV specific immunity in controlling HCMV infection in allogeneic stem cell transplant patients by prospectively monitoring HCMV specific CD4+ and CD8+ T cell function following transplantation.
4. To characterise the CD4+ T cell response to HCMV and identify HCMV specific class II epitopes to the HCMV proteins pp65 and IE1 in HCMV seropositive healthy controls and stem cell transplant recipients for the purposes of targeted monitoring of HCMV specific CD4+ T immune recovery and as possible antigens for generating T-cell clones for immunotherapy and use as a peptide vaccine.

Chapter 2 Risk factors for HCMV Viraemia in Allogeneic Stem Cell Transpiant Recipients in the Era of Pre-emptive Therapy

2.1 Introduction

In order to reduce the risk of symptomatic HCMV infection, the goal is to either avoid HCMV infection altogether by the use of prophylaxis, or to pre-emptively treat asymptomatic infection to prevent viraemia reaching levels that will cause disease. Although prophylaxis is effective in preventing HCMV viraemia and disease when used in the first one hundred days following transplantation (Boeckh et al., 1996), it does not reduce overall mortality, due to increased bacterial and fungal infections secondary to ganciclovir induced myelosuppression negating the reduction in HCMV mortality. Furthermore, prophylactic administration of ganciclovir delays the recovery of HCMV specific immune reconstitution (Li et al., 1994), increasing the risk of late disease. The alternative strategy of pre-emptive therapy based on viral monitoring either by antigenaemia or PCR methods has the advantage of preventing HCMV disease while minimising exposure to myelosuppressive and nephrotoxic drugs.

Despite the use of HCMV surveillance and pre-emptive therapy, problems with late infection remain (Peggs et al., 2000; Einsele et al., 1995). Previously identified risk factors for late HCMV infection include; HCMV antigenaemia before three months, lymphopaenia (absolute lymphocyte count less than 100 X 10^9/L), undetectable HCMV specific T cell

response, and GVHD, with a frequency of late disease among seropositive patients of up to 17.8 percent and a mortality of 46 percent (Boeckh et al., 2003; Boeckh and Nichols, 2004; Peggs et al., 2000). GVHD following allogeneic transplantation is also a significant cause of mortality and impaired quality of life following allogeneic stem cell transplantation. The risk of GVHD can be reduced by depleting T-cells from the graft with Campath-1, a monoclonal antibody against CD52, an antigen found on T cells, B cells, NK cells and dendritic cells (Hale et al., 1983). The rat IgM form, Campath-1M, is effective at depleting donor T-cells from the graft and reducing the risk of GVHD when used ex vivo with autologous serum as a source of complement (Waldmann et al., 1984). However, this technique has been associated with an increased risk of graft rejection, probably mediated by host T-cells (Bunjes, 2000). To overcome this, Campath-1G (IgG rat form) can be used in vivo to deplete host T-cells prior to infusion of the graft, relying on an Antibody Dependent Cell-mediated Cytotoxicity (ADCC) in vivo (Hale et al., 1998), and is effective in reducing acute but not chronic GVHD (Willemze et al., 1992). More recently a recombinant humanised form of anti-CD52, Campath-1H (Alemtuzumab) has been developed and this has superseded the use of Campath-1M and 1G both for ex-vivo and in vivo use (Williams et al., 2000; Phillips et al., 2001). The use of Campath-1H in the setting of non-myeloablative transplant has been reported to be associated with an increased frequency of HCMV infection when used in the non-myeloablative transplant setting, while RIC not utilising

monoclonal antibodies show no differences (Chakrabarti et al., 2002a; Junghanss et al., 2002).

At the Royal Free Hospital, a strategy of prophylactic high dose acyclovir and pre-emptive therapy for HCMV viraemia detected by PCR has been used to prevent HCMV disease. The extensive experience in the use of both Campath-1G and Campath-1H for in vivo and ex vivo T-cell depletion in allogeneic stem cell transplantation allows an assessment of the impact of these agents on HCMV infection in stem cell transplant recipients.

2.2 Methods

2.2.1 Patients

All patients receiving an allogeneic stem cell transplant between 1st of January 1995 and 31st of December 2000 were retrospectively identified. Patient characteristics including age, gender, indication for transplant, disease status at the time of transplant, donor type, stem cell source, degree of HLA match, donor and recipient HCMV serostatus, conditioning regimen, form of T-cell depletion as well as the clinical outcome were collected using the clinical notes and checked against the transplant database. Risk stratification was performed on the basis of underlying disease according to standard criteria *(Glucksberg et al., 1974)*. An underlying diagnosis of CLL was classified as high risk. Patients were regarded as being a complete HLA match if they were identical at Class I A, B or Class II DR by the highest resolution tissue typing available at the

time of transplant. Peripheral blood HCMV PCR results were obtained from the laboratory database.

2.2.2 Conditioning and GVHD Prophylaxis

Standard conditioning regimens were used depending on the indication for transplantation. Non-myeloablative transplants were non-TBI based, using fludarabine, Campath and either busulfan, melphalan, or BEAM (carmustine, etoposide, cytosine arabinoside and melphalan), depending on the underlying condition. Second transplants were conditioned with a fludarabine, cytosine arabinoside and idarubicin based regimen as described previously (Pawson et al., 2001). Non T-cell depleted transplants, with the exception of syngeneic transplants, received short course methotrexate and ciclosporine for GVHD prophylaxis. In the case of T-cell depleted transplants, GVHD prophylaxis with ciclosporine was only used for HLA mismatched or aplastic anaemia transplants. Ex-vivo T-cell depletion was performed by the addition of 20mg of Campath to the stem cell graft for a minimum of 30 minutes at room temperature prior to infusion into the recipient. Campath-1G was used prior to 15th October 1998, and Campath-1H subsequent to 15th October 1998. In 7 cases ex-vivo T-cell depletion was performed by positive CD34 selection using the Baxter Isolex system (Dreger et al., 1995). In-vivo T-cell depletion was performed by the administration of Campath (Campath1G prior to 15th October 1998 and 1H post 15th October 1998) to the transplant recipient prior to transplantation. The standard regimen consisted of 20mg Campath 1G or Campath 1H administered intravenously daily for 5 days prior to infusion of the graft, with a dose reduction for paediatric patients.

Radiotherapy when used in the conditioning regimen (N=152) was TBI at a dose of 750 centiGy target dose, with the exception of two patients who received fractioned TBI (six fractions over three days). In addition, eight patients received Total Lymphoid Irradiation (TLI), and five patients with Fanconi anaemia received Thoraco Abdominal (TA) irradiation.

2.2.3 Infection Prophylaxis

All patients were nursed in positive pressure HEPA (High efficiency particulate air filter) filtered rooms. Ciprofloxacin, amphotericin suspension, and itraconazole or fluconazole were commenced pre-transplant. Pneumocystis pneumonia prophylaxis consisted of nebulized pentamidine until engraftment, then oral trimethoprim/sulfamethoxazole 480 mg twice daily two days per week (dose adjusted for paediatric patients). All HCMV seropositive transplant recipients and seronegative recipients with an HCMV seropositive donor received aciclovir 10mg/kg intravenously three times daily during their inpatient stay, followed by aciclovir 800mg four times daily or valaciclovir 2 g four times daily until 100 days post transplant. Doses were modified in the presence of renal impairment. Otherwise, aciclovir 200mg three times daily was administered as prophylaxis for Herpes Simplex Virus (HSV) if HSV serology was positive. All HCMV seronegative stem cell transplant recipients received HCMV seronegative and leucodepleted blood products, irrespective of donor HCMV serostatus.

2.2.4 HCMV PCR Monitoring

DNA was extracted from whole blood using the QiaAMP DNA Blood minikit (Qiagen). Qualitative HCMV PCR was performed using an in-house PCR assay (Kidd et al., 1993). The lower limit of sensitivity for this assay is 200 genome copies/ml of blood. Stem cell transplant recipients underwent HCMV PCR monitoring twice weekly as an inpatient, then weekly for the first month following discharge, and fortnightly thereafter. Monitoring was continued for a minimum of three months.

2.2.5 HCMV Pre-emptive Therapy

HCMV pre-emptive therapy was commenced after two consecutive HCMV PCR positive results. HCMV viraemia was treated with either ganciclovir 5mg/kg intravenously twice daily, or foscarnet 90mg/kg intravenously twice daily or a combination of both at half dose. Doses were adjusted in the presence of renal failure according to the manufacturer's guidelines. G-CSF was administered to counteract the bone marrow suppressive effect of ganciclovir. During pre-emptive therapy, aciclovir prophylaxis was discontinued. Pre-emptive HCMV therapy was continued until two consecutive negative HCMV PCR results were obtained. Aciclovir prophylaxis was recommenced when pre-emptive therapy was discontinued.

2.2.6 Clinical Outcomes

The major endpoints of this study were first HCMV infection, late HCMV infection, and HCMV disease. Time to HCMV infection was defined as the

time between transplant and the first of two consecutively positive HCMV PCR results. Late HCMV infection was defined as any infection occurring after day 100, irrespective of whether an infection had occurred prior to 100 days. Other endpoints of the study were overall survival, relapse free survival and time to death. Acute GVHD was graded according to standard criteria (Glucksberg et al., 1974).

2.2.7 Statistical Analysis

Statistical analysis was performed using SPSS version 12.1. Kaplan Meier survival analysis was used to calculate HCMV infection rates, acute GVHD rates, overall survival, and relapse free survival. The null hypothesis of the influence of prognostic factors was tested by the log rank score, and p values were two tailed. Univariable risk analysis for HCMV infection was performed using Cox proportional hazards regression models, and multivariable risk analysis was performed using a backwards selection procedure (Cox, 1972). The null hypothesis for regression analysis was tested by the Wald statistic, and the relative risks were estimated as hazard ratios. Median time to HCMV infection was compared using the Mann Whitney U test.

2.3 Results

2.3.1 Patients and Disease Characteristics

Two hundred and six transplant recipients were identified as having received an allogeneic stem cell transplant between the 1st of January 1995 and 31st of December 2000. Patient and disease characteristics are summarised in Table 2-1. The median age at transplantation was 27y

(range 3 to 60y). The major indications for transplantation were acute myeloid leukaemia (n=61), acute lymphoblastic leukaemia (n=52), chronic myeloid leukaemia (n=30), myelodysplastic syndrome (n=16) and non-Hodgkin's lymphoma (n=12), with a smaller number of patients being transplanted for other malignant (n=12) or non-malignant conditions (n=23). One hundred and eighty-three transplants were a complete 6 out of 6 HLA match and 21 transplants were less than a 6 out of 6 HLA match. One hundred and thirty patients received an HLA identical sibling graft, 8 patients received non-sibling related grafts, and 68 patients received unrelated grafts. The source of the graft was bone marrow in 141 cases and G-CSF mobilised peripheral blood stem cells in 64 cases.

Table 2-1 Characteristics of transplant recipients.

No. of transplants, n	*206*
Median age at transplant, yrs (range)	27 (3-60)
Proportion of males (%)	138 (67%)
Indication for transplant	
Acute Myeloblastic Leukaemia	61 (30%)
Acute Lymphoblastic Leukaemia	52 (25%)
Chronic Myeloid Leukaemia	30 (15%)
Myelodysplastic Syndrome	16 (8%)
Non-Hodgkin's Lymphoma	12 (6%)
Other Malignant Disorder[a]	12 (6%)
Other Non-malignant Disorder[b]	23 (11%)
Risk Group	
Standard risk	86 (42%)
High risk	120 (58%)
Donor Type	
Sibling	130 (63%)
Non-sibling related donor	8 (4%)
Unrelated donor	68 (33%)
HLA Match[c]	
6 Antigen match	183 (89%)
Less than 6 antigen match	21 (10%)
Source of Graft	
Bone marrow	141 (68%)
Peripheral blood stem cell	64 (31%)
Median CD34 dose $X10^6$/kg recipient weight[d] (range)	2.6 (0.2-18.4)
Type of transplant	
Reduced intensity conditioning	12 (6%)
Myeloablative	194 (94%)
Pre-transplant conditioning	
Non radiotherapy based conditioning	41 (20%)
Radiotherapy based conditioning	165 (80%)
Campath In Vivo Use[e]	
No Campath in vivo	105 (51%)
Campath-1G in vivo	44 (21%)
Campath-1H in vivo	52 (25%)
Form of Ex vivo T-Cell Depletion	
No ex vivo T-Cell Depletion	116 (56%)
Campath-1G	43 (21%)
Campath-1H	40 (19%)
CD34 selection	7 (3%)
HCMV serology (recipient/donor)[f]	
Neg/Neg	56 (27%)
Neg/Pos	20 (10%)
Pos/Neg	36 (17%)

[a] Idiopathic myelofibrosis, multiple myeloma, sarcoma,
[b] Aplastic anaemia, Fanconi anaemia, thalassaemia major, metachromic leucodystrophy
[c] HLA tissue typing data unavailable in 2 recipient/donor pairs
[d] CD34 dose unavailable in 17 patients
[e] In-vivo Campath use data unavailable in 5 patients
[f] HCMV serology unavailable in 3 recipient/donor pairs

2.3.2 Transplant Related Outcomes

Forty-one patients experienced acute GVHD of grade II or greater, with an actuarial incidence of 24±3.3% when analysed with death as a competing variable. The incidence of grade II, III and IV acute GVHD was 16.2±2.9% (n = 27), 4.3±1.6% (n = 5) and 4.1±1.5% (n = 5) respectively. After a median follow-up of 528 days, overall survival was 79±2.9% at day 100, 57.1±3.6% at 1 year, and 38.2±4.1% at 3 years. By univariable Cox regression analysis, neither HCMV serostatus of the recipient and/or donor, nor the presence of HCMV infection had a statistically significant effect on the overall survival when the entire cohort was analysed, and by subgroup analysis for HLA-identical sibling transplants and matched unrelated transplants, Table 2.2. Overall survival for patients transplanted for malignant conditions (n=183) was 77.5±3.1% at day 100, 52.8±3.8% at one year and 32.3±4.2% at 3 years, with a progression free survival of 70.9±3.4% at day 100, 40.8±3.7% at one year and 26.1±3.7% at 3 years. Neither HCMV serostatus of the recipient and/or donor or HCMV infection had a statistically significant impact on overall survival or progression free survival in this subgroup.

Table 2-2 Univariable Cox Regression analysis of the impact of HCMV serology and infection on overall survival.

	Relative Hazard	*95% CI*	*p value*
All patients (n = 203)			
Positive recipient HCMV serology	1.20	0.74-1.95	0.47
Positive donor HCMV serology	1.01	0.66-1.55	0.98
HCMV infection	1.15	0.71-1.85	0.57
HLA identical sibling transplants (n = 128)			
Positive recipient HCMV serology	1.05	0.53-2.08	0.9
Positive donor HCMV serology	0.73	0.38-1.41	0.35
HCMV infection	1.23	0.67-2.25	0.51
Matched unrelated transplants (n=68)			
Positive recipient HCMV serology	1.31	0.60-2.85	0.50
Positive donor HCMV serology	0.93	0.50-1.74	0.82
HCMV infection	1.01	0.43-2.39	0.98

2.3.3 HCMV Infection

Median follow-up for HCMV PCR monitoring was 129 days. Sixty-six patients experienced HCMV infection, with a median time to HCMV infection of 39 days post transplant (range 0 to 206 days).

Kaplan Meier survival analysis was performed with respect to time to first HCMV infection post transplant on all 147 patients at risk of HCMV infection based on a positive donor and/or recipient HCMV serology. Overall, by Kaplan Meier analysis the cumulative infection rate was 15.3±3.0% by 1 month (30 days), 40.1±4.2% by 2 months (60 days), 42.4±4.2% by 3 months (100 days), 48.7±4.4% by 6 months (180 days) and 50.7±4.4% by 1 year (365 days) after transplant, Figure 2-1. Kaplan Meier analysis of the incidence of HCMV infection stratified by donor (D) and recipient (R) HCMV serostatus is summarised in Figure 2-2. The cumulative infection rate at 6 months for the R+D- group was 56.3±9.1%, for R+D+ was 55.5±5.5% and for the R-D+ group was 16.9±9%. The difference in the cumulative HCMV infection rates between the R-D+ group, and the R+D- and R+ D+ groups was statistically significant ($p=0.009$ for R+D- group and $p=0.007$ for R+D+ group when compared with the R-D+ group, Log Rank Score). There was no statistically significant difference in infection rates between the R+D- group and the R+D+ group.

No HCMV viraemia was detected in the R-D- group, although one patient in this group did develop HCMV colitis with a positive DEAFF test despite an absence of HCMV viraemia.

Figure 2-1 Kaplan Meier estimate of the cumulative incidence of HCMV infection in intermediate or high risk patients.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection is plotted for recipient and/or donor HCMV seropositive patients (n=147). The cumulative incidence of infection is 15.3±3.0% at 30 days, 40.1±4.2% at 60 days, 42.4±4.2% at 100 days, 48.7±4.4% at 180 days and 50.7±4.4% at 360 days. The cross bars indicate censored events.

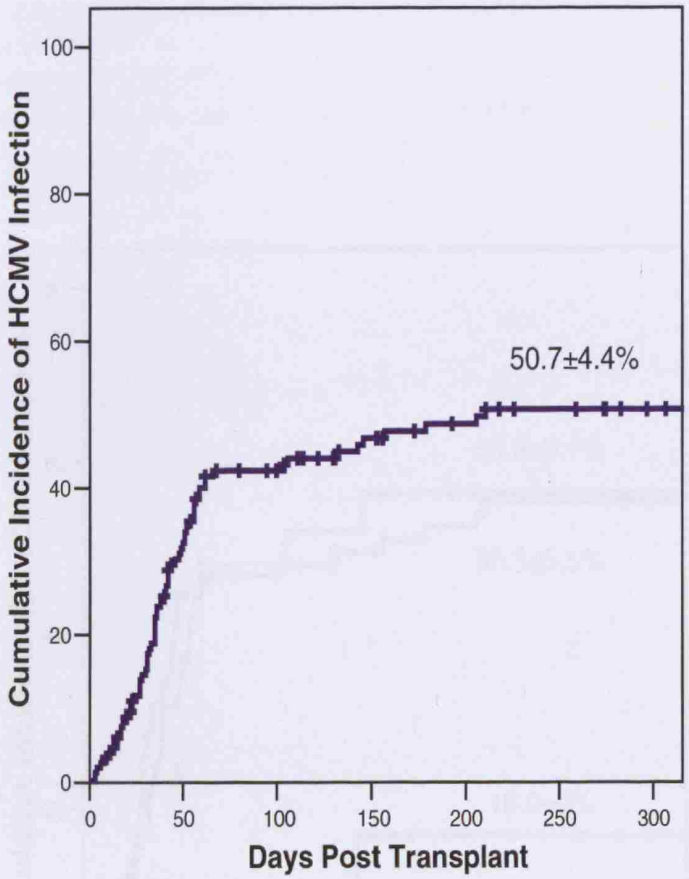
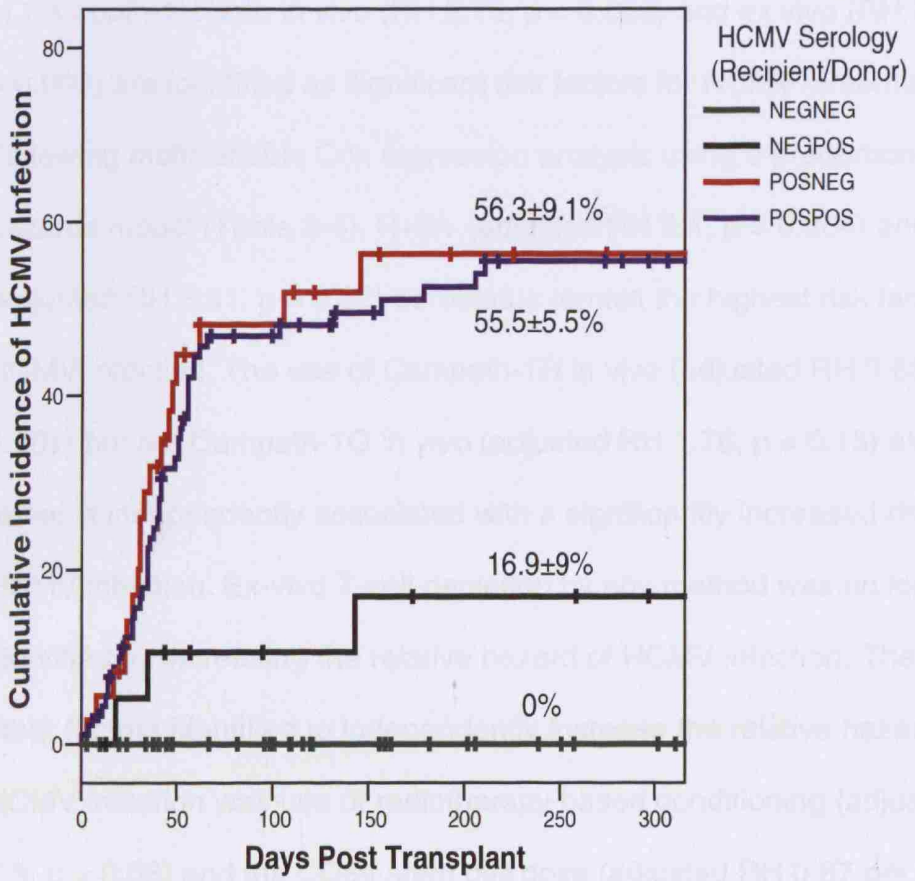


Figure 2-2 Kaplan Meier estimate of the cumulative incidence of HCMV infection according to HCMV serology.

The Kaplan Meier estimates of the cumulative incidence of HCMV infection according to HCMV serology of the recipient and the donor is plotted. The R-D- group (n=56, green line) did not experience any episodes of viraemia. The cumulative incidence of viraemia of the R-D+ group (n=20, black line) was 16.9±9%, the R+D- group (n=36, red line) was 56.3±9.1% and the R+D+ group (n=91, blue line) was 55.5±5.5%. %. The difference in the incidence of HCMV infection between the R-D+ group, and the R+D- and R+ D+ groups was statistically significant (p=0.009 for R+D- group and p=0.007 for R+D+ group when compared with the R-D+ group, Log Rank Score). There was no statistically significant difference in infection rates between the R+D- group and the R+D+ group (p=0.71, Log Rank Score). The cross bars indicate censored events.



2.3.4 Analysis of the Risk Factors for HCMV Infection

The results of univariable Cox regression analysis for the risk factors for HCMV infection in patients at risk of HCMV viraemia are summarised in Table 2-3. Consistent with the results of the cumulative incidence of HCMV infection by Kaplan Meier analysis, univariable Cox regression analysis shows that R+D+ serostatus (RH 4.28, p = 0.02) and R+D- serostatus (RH 4.73, p=0.02) are associated with the greatest relative hazards for HCMV infection. Among the other factors examined, the use of Campath-1H both in vivo (RH 2.15, p = 0.006) and ex vivo (RH 2.11, p = 0.009) are identified as significant risk factors for HCMV viraemia. Following multivariable Cox regression analysis using a proportional hazards model (Table 2-4), R+D+ (adjusted RH 8.1, p = 0.004) and R+D- (adjusted RH 5.91, p = 0.02) serostatus remain the highest risk factors for HCMV infection. The use of Campath-1H in vivo (adjusted RH 3.68, p < 0.001) but not Campath-1G in vivo (adjusted RH 1.76, p = 0.15) also remains independently associated with a significantly increased risk of HCMV infection. Ex-vivo T-cell depletion by any method was no longer identified as increasing the relative hazard of HCMV infection. The only other factors identified to independently increase the relative hazard of HCMV infection was use of radiotherapy based conditioning (adjusted RH 2.3, p = 0.03) and the CD34 stem cell dose (adjusted RH 0.87 per $1X10^6$/kg CD34 cells, p = 0.04), while age was no longer significant.

Table 2-3 Univariable Cox Regression analysis of the risk factors for HCMV infection.

		Relative Hazard	*95% CI*	*p value*
Age	per year	1.02	1.00-1.03	0.08
Gender	Female	1.12	0.68-1.83	0.66
Risk	High Risk	0.87	0.54-1.41	0.58
Campath In Vivo	No Campath	1.00		0.024
	Campath-1G	1.36	0.69-2.68	0.38
	Campath-1H	2.15	1.24-3.73	0.006
Campath Ex Vivo	No Campath	1.00		0.06
	Campath-1G	1.04	0.54-1.99	0.91
	Campath-1H	1.97	1.06-3.66	0.009
	CD34 Selection	0.00		0.90
Acute GVHD	GVHD grade II or higher	1.25	0.70-2.22	0.45
Pre-transplant conditioning	Radiotherapy based conditioning	1.97	0.97-3.97	0.06
Donor Type	Sibling	1.00		0.44
	Unrelated	1.34	0.79-2.29	0.28
	Non Sibling Related Donor	0.67	0.16-2.77	0.58
Donor Match	Fewer than 6 antigen match	1.10	0.44-2.75	0.83
BMT Type	Reduced intensity	0.83	0.25-2.56	0.71
Graft Source	PBSC	0.90	0.54-1.52	0.70
CD34 dose	per 10^6/kg recipient weight	0.90	0.80-1.01	0.08
HCMV serology	NegPos	1.00		0.04
(Recipient/Donor)	PosNeg	4.73	1.39-16.1	0.02
	PosPos	4.28	1.33-13.74	0.02
Disease	ALL	1.00		0.94
	AML	0.90	0.46-1.75	0.75
	CML	1.22	0.58-2.61	0.59
	MDS	1.07	0.45-2.95	0.77
	Other Malignant	0.81	0.35-1.90	0.64
	Other Non-Malignant	0.90	0.37-2.20	0.82

Table 2-4 Multivariable Cox Regression analysis of the risk factors for HCMV infection.

		Relative Hazard	*95% CI*	*p value*
Campath In Vivo Use	No Campath	1.00		<0.001
	Campath-1G	1.76	0.81-3.84	0.15
	Campath-1H	3.68	2.02-6.72	<0.001
Pre-transplant Conditioning	Radiotherapy based conditioning	2.30	1.07-4.91	0.03
CD34 dose	per 10^6/kg recipient weight	0.87	0.77-0.99	0.04
HCMV serology	NegPos	1.00		0.02
(Recipient/Donor)	PosNeg	5.91	1.34-26.06	0.02
	PosPos	8.10	1.93-33.91	0.004

2.3.5 Effect of In Vivo Campath Use on Time to HCMV Infection

Kaplan Meier survival curves for HCMV infection stratified by use of Campath in vivo were plotted for all patients at risk of HCMV viraemia, (Figure 2-3). The cumulative HCMV infection rate for patients receiving Campath-1H in vivo was 64.7±8.6%, for patients receiving Campath-1G in vivo was 41.6±10.4% and for patients receiving no Campath-1 in vivo was 42.8±5.8%. The difference in the cumulative incidence of HCMV infection between the Campath-1H group and the non-Campath group was highly significant (p=0.0024, Log Rank Score), but the difference between the Campath-1G group and the Campath-1H group did not reach statistical significance. Although the group receiving no Campath in vivo had a lower incidence of infection prior to day 100 (cumulative incidence of 52.2±5.3%), the overall cumulative incidence was comparable to the Campath-1G group.

To assess the interaction between HCMV serostatus and the effects of Campath in vivo, separate Kaplan Meier survival curves according to in vivo Campath use were plotted for R+D+ patients, (Figure 2-4) and R+D- patients, (Figure 2-5). In the R+D+ group, although HCMV infection occurs earlier in the groups receiving either Campath-1H in vivo (cumulative incidence 63.9±12.9%) or Campath-1G in vivo (cumulative incidence 58.4±15.6%) compared to patients receiving no Campath in vivo (cumulative incidence 49.7±6.7%), there is no significant difference in the overall cumulative incidence of HCMV infection (p = 0.15, Log Rank Score). In the R+D- group, the cumulative incidence of HCMV infection is 100% for Campath-1H in vivo, 46±17.3% for Campath-1G in vivo and

25±12.7% for no Campath. This difference is statistically significant ($p < 0.001$, Log Rank Score). In the R-D+ group, the cumulative incidence of HCMV infection among patients receiving Campath-1H in vivo was 16.7±15.2%, and among patients not receiving Campath in vivo was 14.3±13.2%, while none of the patients that received Campath-1G in vivo experienced infection. The difference was not significant ($p=0.72$, Log Rank Score).

When the cumulative incidence of infection between R+D- and R+D+ patients was compared in a subgroup analysis according to Campath in vivo use, none of the groups showed a statistically significant difference ($p=0.13$ for no Campath group, $p=0.61$ for Campath-1G in vivo group and $p=0.24$ for Campath-1H in vivo group, Log Rank Score).

HCMV infection occurred significantly earlier in patients receiving either Campath-1G in vivo, with a median time to infection of 27 days, or Campath-1H in vivo with a median time to infection of 33 days, when compared to patients receiving no Campath in vivo, median time to infection of 51 days ($p=0.007$ for Campath-1G vs. no Campath and $p=0.006$ for Campath-1H vs. no Campath, Mann Whitney U Test), Figure 2.6. The difference in the time to HCMV infection between the Campath-1G and the Campath-1H group was not statistically significant ($p=0.97$, Mann Whitney U Test).

Figure 2-3 Kaplan Meier estimate of the cumulative incidence of HCMV infection according to Campath in vivo use.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection in recipient and/or donor HCMV seropositive patients, according to in vivo Campath use is plotted. The cumulative incidence of HCMV infection in the group receiving no Campath in vivo (red line, n=85) was 42.8±5.8%, for the group receiving Campath-1G in vivo (blue line, n=28) was 41.6±10.4%, and for the group receiving Campath-1H (green line, n=32) was 64.7 ±8.6%. The difference in the cumulative incidence of infection between the Campath-1H group and the no Campath group was significant (p=0.002, Log Rank Score), while the difference between the group receiving Campath-1G and no Campath group (p=0.71, Log Rank Score), and Campath-1G and Campath-1H (p=0.13, Log Rank Score) was not significant. The cross bars indicate censored data.

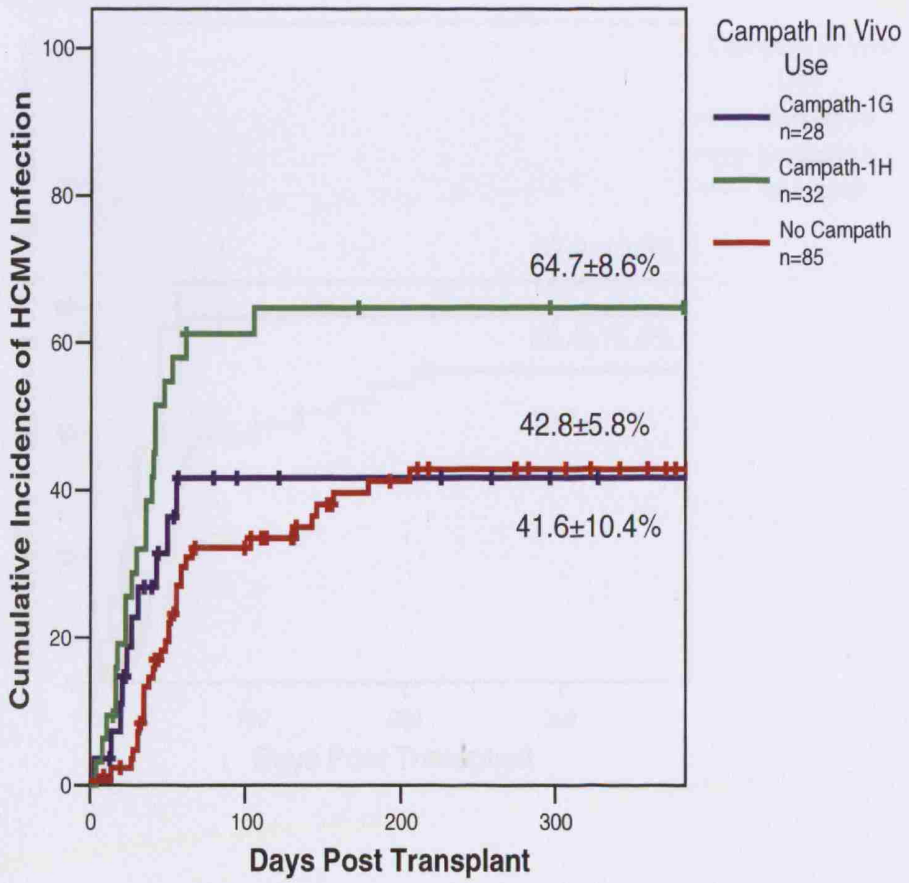


Figure 2-4 Kaplan Meier estimate of the cumulative incidence of HCMV infection in R+D+ patients according to Campath in vivo use.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection in R+D+ allogeneic transplant patients according to the in vivo use of Campath. The cumulative incidence of HCMV infection for the group not receiving Campath in vivo (n=63, red line) is 49.7±6.7%, for the group receiving Campath-1G in vivo (n=11) is 58.4±15.6%, and for the group receiving Campath-1H is 63.9±12.9%. There was no statistically significant difference between the curves (p=0.15, Log Rank Score). The cross bars indicate censored data.

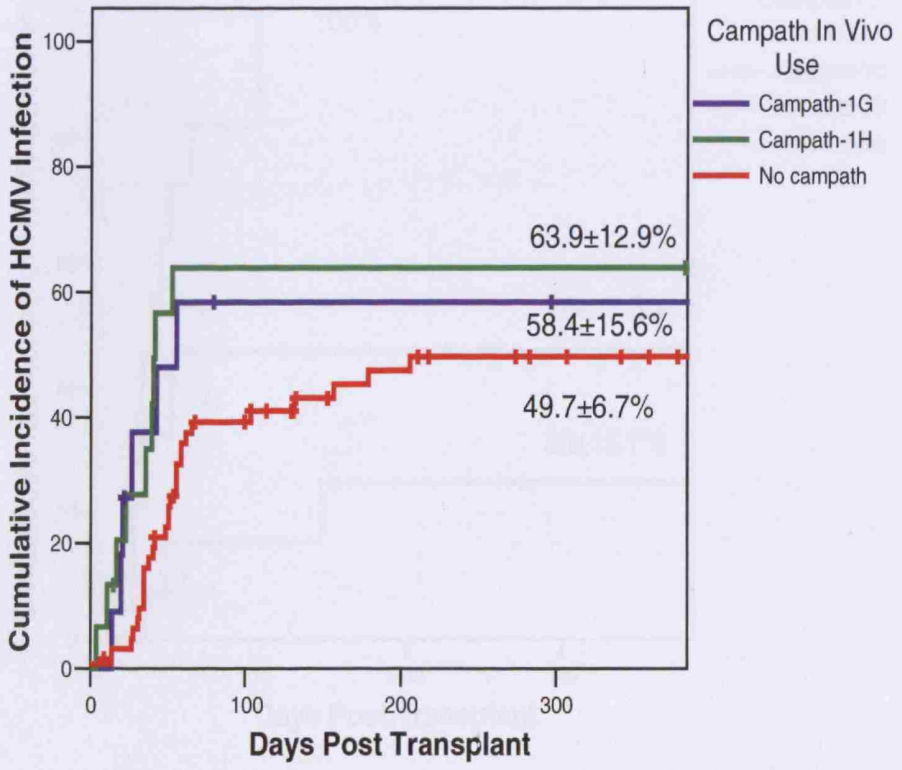


Figure 2-5 Kaplan Meier estimate of the cumulative incidence of HCMV infection in R+D- patients according to Campath in vivo use.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection in the R+D- group is plotted according to in vivo Campath use. The cumulative incidence of HCMV infection in the no Campath group (red line, n=15) was 25±12.7% and the Campath-1G group (blue line, n=10) was 46±17.3%. All the non-censored patients in the Campath-1H group (green line, n=10) experienced HCMV infection. The difference between the curves was statistically significant (p=0.0005, Log Rank Score). The cross bars indicate censored data.

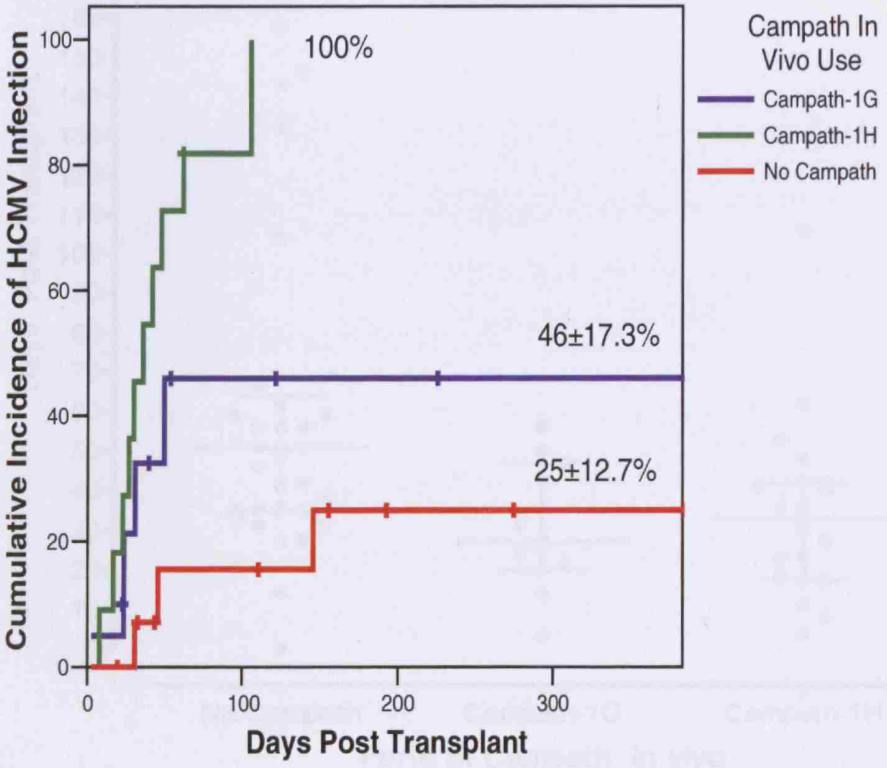
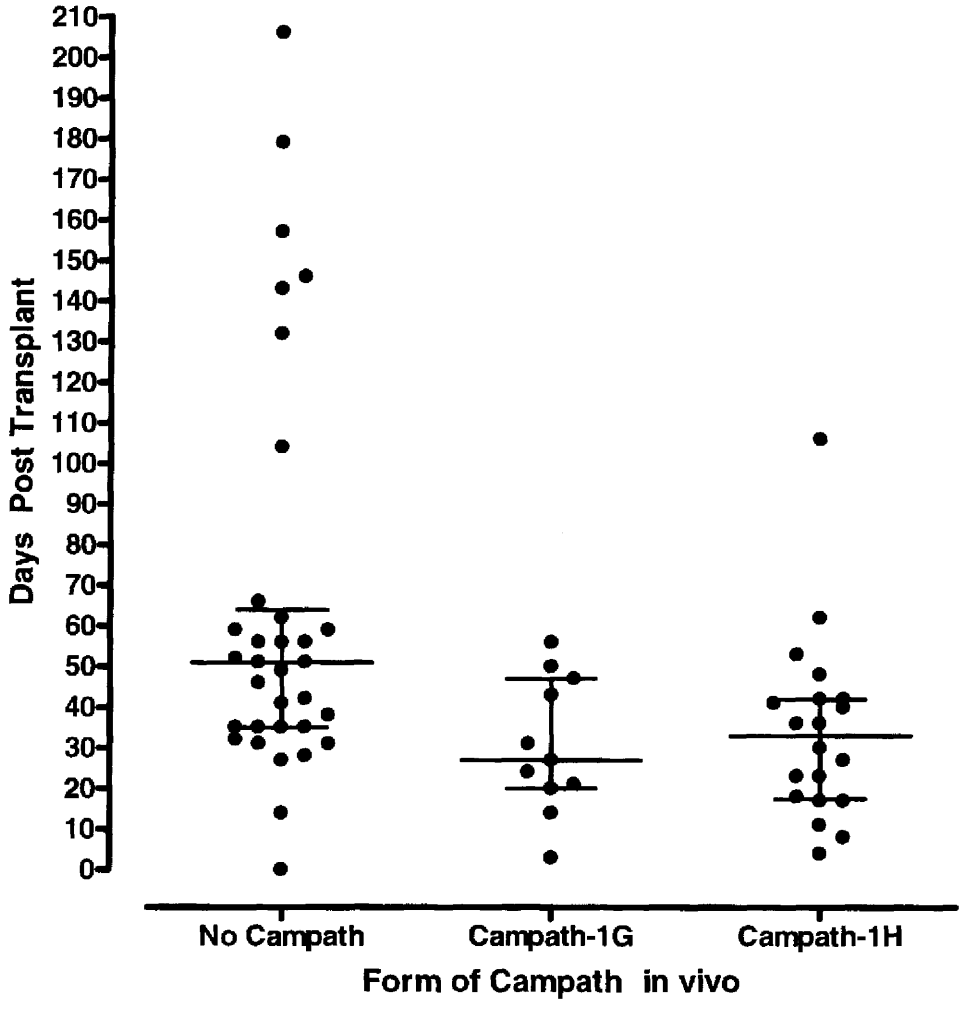


Figure 2-6 Time to first HCMV infection according to Campath in vivo use.

The time to first HCMV infection following transplantation is plotted for allogeneic recipients according to Campath in vivo use. The horizontal bar indicates the median value and the interquartile range. The median time to HCMV infection in the group not receiving Campath in vivo (n=33) was 51 days (range 0 to 206 days), for the group receiving Campath-1G (n=11) was 27 days (range 3 to 56 days), and for the group receiving Campath-1H (n=20) was 33 days (range 4 to 106 days). There was a significant difference in time to first HCMV infection between the Campath-1G group and the non Campath group (p=0.007, Mann Whitney U Test), and between the Campath-1H group and the non Campath group (p=0.006, Mann Whitney U Test). The difference between the Campath-1G and Campath-1H group was not significant (p=0.97, Mann Whitney U Test).



2.3.6 Late HCMV Infection

One hundred and seventeen patients with follow-up beyond 100 days were identified and included in the analysis of risk factors for late HCMV infection. Twenty patients experienced HCMV infection beyond 100 days. The Kaplan Meier estimate of the incidence of late HCMV viraemia is 19.5±5.4%, Figure 2-7. There was no statistically significant difference in the incidence of late HCMV infection according to HCMV serostatus, Figure 2-8.

Univariable Cox regression analysis of the risk factors for late HCMV infection is shown in Table 2-5. Infection before day 100 (RH 2.5, p=0.05) was the only significant factor predictive of the risk of late infection. However, following multivariable Cox regression analysis, increasing age (adjusted RH 1.04 per year p = 0.04) and an acute GVHD score of II or greater (adjusted RH 4.0, p = 0.01) were both statistically significant predictors of an increased risk of late HCMV infection, while a higher CD34 stem cell dose was associated with a reduced risk of late infection (RH 0.8 per $1X10^6$ CD34 cells/kg recipient weight, p = 0.05). These data are summarised in Table 2-6.

Table 2-5 Univariable Cox regression analysis of the risk factors for late (>100 days) HCMV infection.

		Relative Hazard	*95% CI*	*Significance (p value)*
Age	per year	1.03	1.00-1.06	0.07
Gender	Female	1.10	0.44-2.76	0.84
Risk	High Risk	0.91	0.38-2.19	0.84
Campath In Vivo use	Campath-1G	1.11	0.31-3.94	0.87
(Reference group is No Campath)	Campath-1H	1.06	0.36-3.02	0.91
Campath Ex Vivo Use	Campath-1G	2.05	0.70-6.02	0.19
(Reference group is No Campath)	Campath-1H	1.46	0.50-4.26	0.49
	CD34 Selection	0	0-∞	0.98
Acute GVHD	GVHD grade II or higher	2.27	0.87-5.93	0.09
Pre-transplant Conditioning	Radiotherapy based conditioning	5.68	0.76-42.43	0.09
Donor Type	Unrelated	1.47	0.57-3.83	0.43
	Non Sibling Related Donor	0	0-∞	0.98
Donor Match	Fewer than 6 antigen match	1.21	0.16-9.06	0.85
BMT Type	Reduced intensity	1.50	0.20-11.23	0.69
Graft Source	PBSC	0.97	0.37-2.53	0.95
CD34 dose	per 10^6/kg recipient weight	0.81	0.62-1.04	0.1
HCMV serology (Recipient/Donor)	PosNeg	1.93	0.39-9.54	0.42
(Reference group is Neg/Pos)	PosPos	1.27	0.29-5.69	0.75
Disease	ALL	1		0.53
	AML	1.41	0.41-4.83	0.58
	CML	1.03	0.19-5.59	0.98
	MDS	2.96	0.66-13.24	0.16
	Other Malignant Disorder	0.70	0.08-6.23	0.75
	Other Non-Malignant Disorder	0.42	0.05-3.75	0.44
HCMV Infection Before Day 100	Yes	2.47	1.01-6.05	0.05

Table 2-6 Multivariable Cox Regression analysis of the risk factors for late (>100 days) HCMV Infection.

		Relative Hazard	*95% CI*	*Significance (p value)*
Age	per year	1.04	1.00-1.09	0.04
Acute GVHD	GVHD grade II or higher	3.96	1.39-11.30	0.01
Radiotherapy	Radiotherapy based conditioning	6.06	0.60-61.71	0.13
BMT Type	Reduced intensity	10.11	0.87-117.54	0.07
CD34 dose	per 10^6/kg recipient weight	0.78	0.61-0.99	0.05

Figure 2-7 Kaplan Meier estimate of the cumulative incidence of late HCMV infection.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection after day 100 post transplant is plotted for patients at risk of HCMV infection and with follow-up beyond 100 days (n=117). The cumulative incidence of infection was 19.5±4%. The cross bars indicate censored data.

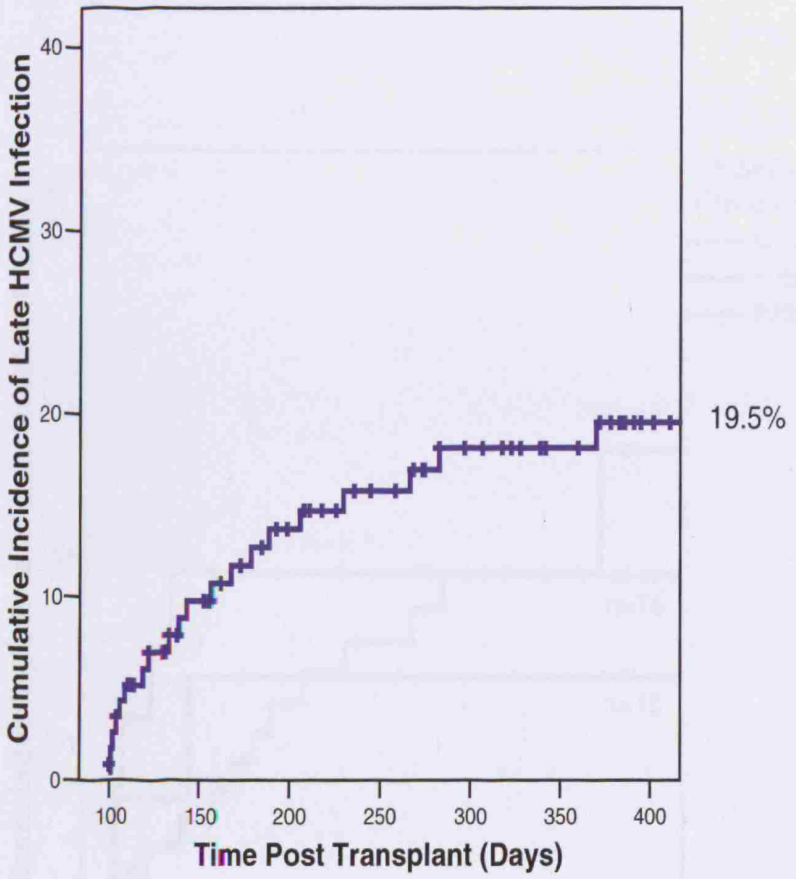
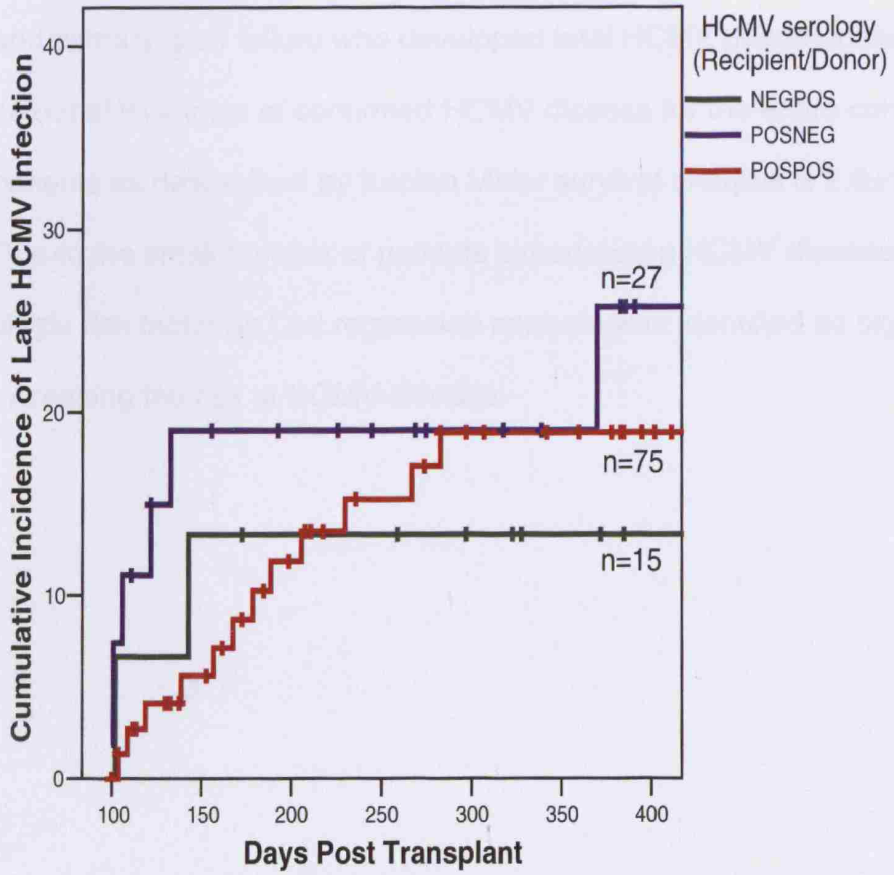


Figure 2-8 Kaplan Meier incidence of late HCMV infection according to HCMV serology.

The Kaplan Meier estimate of the cumulative incidence of HCMV infection occurring after one hundred days is plotted for patients at risk of HCMV infection and follow-up beyond 100 days. The cumulative incidence for R+D- patients (n=27, blue line) is 25.8±9.6%, for R+D+ patients (n=75, red line) is 18.9±5%, and for R-D+ patients (n=15, green line) is 13.3±8.9%. The cross bars indicate censored data. There was no statistically significant difference in the curves by the Log Rank Test.



2.3.7 HCMV Disease

In this cohort of patients, there were five confirmed cases of HCMV disease, including three cases of HCMV colitis, one case of HCMV hepatitis, and one case of HCMV pneumonitis. There were an additional three cases of probable HCMV disease that did not fulfil the current working group definitions of HCMV disease. There was one confirmed death due to HCMV disease, in a patient with myelodysplastic syndrome and primary graft failure who developed fatal HCMV pneumonitis. The actuarial incidence of confirmed HCMV disease for the entire cohort of patients as determined by Kaplan Meier survival analysis is 2.6±1.2%. Due to the small number of patients experiencing HCMV disease, no single risk factor by Cox regression analysis was identified as significantly increasing the risk of HCMV disease.

2.4 Conclusions

The efficacy of aciclovir prophylaxis, combined with HCMV PCR surveillance and pre-emptive therapy is confirmed by the fact that there was a very low incidence of HCMV disease. The absence of HCMV viraemia in the R-D- group confirms the effectiveness of using HCMV seronegative blood products to prevent HCMV infection in these patients. The small number of HCMV infections in the R-D+ group most probably represents primary infections acquired from the donor. This observation and the absence of a significant difference in infection rates between the R+D+ and R+D- groups suggests that the majority of infections in seropositive recipients are due to reactivation of latent virus. A positive donor serology was not found to be protective for HCMV infection in this study, contrary to what has been shown previously (Grob *et al.*, 1987). In the case of patients not receiving Campath in vivo, the incidence of HCMV infection was in fact higher for R+D+ patients (49.7±6.7%) than R+D- patients (25±15.2%), and while the incidence in R+D- patients receiving Campath-1H (100%) was higher than R+D+ patients receiving Campath-1H (63.9±12.9%), neither of these differences was found to be significant.

In this study, HCMV seropositive donor and/or recipient HCMV had no impact on overall survival for the whole group or subgroup analysis for sibling and matched unrelated transplants. This contrasts with the findings of Craddock et al who reported that HCMV recipient seropositivity had an adverse influence on outcome for T-cell depleted matched unrelated transplants for CML using Campath (Craddock et al.,

2001). In addition, Kroger showed a substantially reduced overall survival in HCMV seropositive matched unrelated transplant recipients T-cell depleted with ATG compared to HCMV seronegative patients (Kroger et al., 2001). HCMV seropositivity has also been reported to decrease 5 year overall survival by up to 24% following partially T-cell depleted HLA identical sibling transplants (Broers et al., 2000) due to an increase in treatment related mortality, probably as a result of the myelosuppressive effect of ganciclovir. However, in a large EBMT study, Ljungman showed a beneficial effect of positive donor HCMV serostatus on overall survival and treatment related mortality for matched unrelated donor transplants, although the benefit was not seen with T-cell depletion (Ljungman et al., 2003a). HCMV infection detected by antigenaemia when managed in the context of pre-emptive therapy has been associated with a worse outcome (Yanada et al., 2003), but there was no impact of HCMV PCR positivity on overall survival in this study.

Consistent with previous studies, HCMV seropositivity is confirmed as the most significant risk factor for HCMV infection. In this study, the next most important predictor for HCMV infection was the use of Campath-1H in vivo, which is consistent with the findings of Chakrabarti (Chakrabarti et al., 2002a) who showed that Campath-1H in vivo in the setting of non-myeloablative conditioned transplants increased HCMV infection rates. The finding of an earlier time to HCMV viraemia in recipients of Campath-1H or Campath-1G in vivo compared to patients receiving no Campath in vivo is likely to reflect higher rates of viral replication in the absence of an immune response, suggesting delayed recovery of HCMV specific

immunity. The impact of HCMV specific immunity on viral replication following liver transplantation shows that HCMV seronegative recipients of an HCMV positive organ experience higher rates of HCMV replication than seropositive recipients, due to partial suppression of viral replication by the pre-existing immune response (Emery *et al.*, 2002). The data showing the greater cumulative incidence of infection in R+D- patients receiving Campath-1H in vivo compared to no Campath in vivo suggests that Campath-1H prevents the generation of an HCMV specific immune response, perhaps with a disproportionately greater effect on pre-existing recipient HCMV immunity, and this effect is greater with Campath-1H than Campath-1G.

In contrast to the in vivo use of Campath, ex vivo use of Campath to T-cell deplete the graft did not significantly increase the risk of HCMV infection. Compared to T-cell depletion by positive selection, ex vivo T-cell depletion by Campath results in less removal of T-cells and NK cells (Dreger et al., 1995). The use of Campath "in the bag", particularly the lower dose of 10mg has been shown to effectively decrease the incidence of graft versus host disease without compromising immune recovery (Chakrabarti et al., 2003; Novitzky et al., 2004).

In this study there was a trend towards a higher incidence of HCMV infection with Campath-1H compared to Campath-1G, and this is probably be due to the difference in half lives between the two forms of Campath. Following the administration of 20 mg of Campath-1H for five days (-8 to -4), the antibody remains detectable 28 days after transplant (Morris et al., 2003), and it is likely that this is a significant factor in

delaying HCMV specific T-cell immune reconstitution compared to patients receiving no Campath in vivo. Campath-1G has a significantly shorter half life, of approximately 24 hours (Rebello et al., 2001), and when used in vivo, effectively depletes host dendritic cells without delaying donor dendritic cell reconstitution (Klangsinsirikul et al., 2002). There was a steady increase in the cumulative incidence of first episode of HCMV infection up to day 100, followed by a further slower increase after day 100. It is likely that there are different factors predisposing patients to HCMV infection before and after 100 days. Age, an acute GVHD score of II or greater and a low CD34 dose were identified as the most significant predictors of late HCMV infection. However, the study of risk factors for late HCMV infection are susceptible to selection bias as only patients felt to be at increased risk of HCMV infection were monitored beyond 100 days.

An acute GVHD score of II or greater was only found to be a risk factor for HCMV infection beyond the first 100 days among those high risk patients selected for extended monitoring, and is is likely to be due to the use of immunosuppressive therapy, including corticosteroids. A Japanese study by Yanada (Yanada et al., 2003) found that acute GVHD (grades II-IV) was the only significant factor to predict HCMV infection on multivariate analysis. Allogeneic reactions are known to stimulate HCMV from a latent state into the replicative state (Soderberg-Naucler et al., 1997), and the use of steroid to treat GVHD, further suppresses immune function, allowing the viral replication rate to increase. The overall frequency of acute GVHD in this study was low, probably due to the

extensive use of T-cell depletion with Campath, and this may account for the finding that an acute GVHD score of greater than I did not significantly increase the risk of HCMV infection in the first 100 days.

Non-myeloablative transplant regimens also showed a trend towards a greater frequency of late infection in this study, independent of the effect of Campath, but due to the relatively small number of such transplants in this cohort as well as selection bias, this study is not adequately powered to answer this question. In matched RIC sibling allogeneic transplant recipients using ATG based conditioning, bone marrow as a source of stem cells is associated with an increased risk of HCMV infection (Mohty et al., 2003b). The Seattle group have shown similar findings in the non-T cell depleted setting, (Hakki et al., 2003), as well as delayed CD4 and CD8 recovery. However, the Seattle group found the incidence of HCMV infection in RIC transplants using mycophenolate and low dose (2Gy) radiotherapy at 365 days was similar to conventional intensity conditioned transplants but a later time to infection (Junghanss et al., 2002). Thus, prolonged monitoring for HCMV should be performed in selected patients, including RIC transplants, older transplant recipients, patients with GHVD, and patients with poor CD34 counts.

Chapter 3 HCMV Replication Kinetics

3.1 Introduction

One of the main risk factors for the development of HCMV disease in the immunosuppressed patient is the peak viral load attained during viraemia. This is true in HIV (Spector et al., 1998), renal transplantation (Cope et al., 1997; Hassan-Walker et al., 1999), liver transplantation (Humar et al., 1999; Mendez et al., 1998) and stem cell transplantation (Cope et al., 1997; Gor et al., 1998). However, these studies were performed by retrospectively quantifying HCMV viral loads by quantitative competitive PCR and did not utilise pre-emptive therapy for HCMV infection. Furthermore, by the time peak viral load is attained, disease is already present. Thus, in order to prevent HCMV disease, the goal should be to prevent high levels of HCMV viraemia by using a sensitive surveillance technique to initiate pre-emptive therapy for infection. In addition, the identification of patients at risk of high viral loads on the basis of viral replication kinetics can be envisaged (Emery et al., 2000). A model of viral replication established initially for HIV and applicable to HCMV infection shows that the main determinants of peak viral load are the rate of viral replication, the number of target cells present, the rate of infected target cell death and the rate of clearance by either the immune system or antiviral therapy, and this can be mathematically predicted, as shown in Equation 3.1 (Bonhoeffer et al., 1997).

Equation 3-1 Model for HCMV Replication.

$$dI/dt = \beta IT - \delta_I I - \rho IE$$

I = the number of infected cells, t = time, β = the rate of new infections occurring in uninfected cells, T = target cell number, δ_I = death rate of infected cells, ρ = proportion of infected cells undergoing CTL lysis and E = number of HCMV-specific effector CTLs.

In contrast to the rate of viral replication in fibroblast culture, replication of HCMV in vivo is rapid with a doubling time of 24 hours in individuals with impaired immunity (Emery et al., 1999). When viral loads are examined in solid organ and stem cell transplant recipients, both the initial viral load at the time of a first PCR positive result, and the initial rate of viral replication are predictive of the risk of HCMV disease (Emery et al., 2000). Using the initial rate of rise in viral load, it is also possible to calculate the Ro or the basic reproductive number of HCMV, which is a measure of the number of infected cells produced from a single HCMV infected cell before target cells are depleted. This value can be used to infer the level of HCMV immunity, as has been shown in a comparison of HCMV infection in immune and non immune liver transplant recipients (Emery et al., 2002), where the doubling time of HCMV reflects pre-existing HCMV immunity.

Viral dynamics can also be used to evaluate the efficacy of antiviral therapy. In solid organ transplant recipients, HCMV viral load dynamics, specifically the time to viral clearance and the half life of decline in viral load are significantly correlated with the risk of recurrence (Humar et al.,

2002), with viral load kinetics following a logarithmic decay according to the calculation, Equation 3.2.

Equation 3-2 Logarithmic decay curve of viral load following initiation of antiviral therapy.

$$y=y_0e^{-ax}$$

y_0=initial viral load, a=decay constant and x=time from start of treatment.

Since the incidence of HCMV disease has fallen considerably with the advent of HCMV monitoring and pre-emptive therapy, viral dynamics can also be used as a surrogate marker for comparing the efficacy of treatments such as ganciclovir and combination ganciclovir/foscarnet. The feasibility of this approach has already been established in the liver and renal transplant setting, with the demonstration of equivalence in efficacy of ganciclovir and valganciclovir in controlling viraemia (Mattes et al., 2005).

With the advent of real time PCR techniques such as TaqMan and LightCycler it is now possible to monitor the viral load with a very short laboratory turnaround time. However, the impact of viral dynamics using a real time PCR monitoring strategy on pre-emptive therapy strategies has not been fully assessed.

3.2 Methods

3.2.1 Patients

Allogeneic stem cell transplant recipients were prospectively monitored for HCMV infection by twice weekly blood samples whilst an inpatient

then at each outpatient appointment. All patients at risk of HCMV infection received high dose aciclovir prophylaxis.

Prior to 1/7/02, patients with HCMV infection (based on two consecutive HCMV PCR positive results) were treated either with ganciclovir (5mg/kg twice daily), valganciclovir 900mg twice daily or combination therapy with ganciclovir (5mg/kg per day) and foscarnet (90mg/kg per day) at the clinician's discretion. For patients receiving ganciclovir therapy, G-CSF 300 µg was administered on alternate days to counteract any neutropaenia due to bone marrow suppression. Antiviral therapy was continued until HCMV PCR was negative on two consecutive occasions. Following the 1st of July 2002, HCMV specific antiviral therapy was initiated if the patient had a single positive PCR result greater than 3000 copies/ml, or two consecutive positive results irrespective of the viral load. Adherence to the protocol was generally good, with only two exceptions identified.

3.2.2 Extraction of DNA from Whole Blood

HCMV PCR was performed on whole blood using citrated blood samples as EDTA and heparinised samples may interfere with the PCR amplification. DNA was extracted using the QIAamp DNA blood extraction kit (Qiagen, UK). 200 µL of a citrated whole blood sample was added to a micro centrifuge tube containing 20 µL of QIAGEN Protease and 200 µL of lyses buffer was added. After pulse vortexing the sample was incubated at 56°C for 10 minutes. 200 µL of 96% ethanol (v/v) was added to the sample and after further pulse vortexing; the mixture was added to a QIAamp spin column containing a silica matrix for adsorption

of the DNA. The spin column was spun at 6000 RPM for 1 minute. The column was washed twice with 500 µL aliquots of Qiagen buffers. The DNA was then eluted by the addition of 200 µL of Qiagen AE buffer. Samples were either quantified immediately or stored at -20°C for later quantification.

3.2.3 Real Time Quantitative PCR

HCMV viral load was determined by real time PCR using a Taqman (ABI) based method. The probe and primer sequences were designed using the Primer Express software (Perkin-Elmer, Applied Biosystems, Foster City, Calif.), and produced by MWG-Biotech AG (Germany).

The sequence for the Glycoprotein B specific CMV primers used was:

gB1 5'-GAGGACAACGAAATCCTGTTGGGCA-3'

gB2 5'-TCGACGGTGGAGATACTGCTGAGG-3'

The gB probe used was a 29-mer Taqman probe labelled with 6-FAM at the 5' end and TAMRA at the 3' end, with the sequence:

5'-CAATCATGCGTTTGAAGAGGTAGTCCACG-3'

The reaction mixture consisted of 2.5 µL of 10x PCR buffer (containing 1.5 mmol/L of $MgCl_2$), 0.5 µL of MgCl (25 mmol/L), 0.75 µL of dNTPs (containing 6.25 mmol/µL of dATP, dCTP, dGTP and dUTP), 1.2 µL of the gB TaqMan probe (5 pmol/µL), 1 µL of gB1 and gB2 primers (15 pmol/µL), 0.25 µL of HotStart Taq DNA polymerase (0.25 IU/µL) and 12.8 µL of sterile water to make a final volume of 20 µL, which was added to each well of an ABI 96 well plate. For the generation of a control curve, 1, 5, 10, 50, 1 X 10^2, 1 X 10^3 and 1 X 10^4 gB genome copies per 5 µL were prepared by serial dilution of a plasmid at 1 X 10^5 genome copies/ml

concentration of the entire gB sequence (courtesy Nigel Temperton) into 10mMol TRIS buffer pH 8.5 containing sheared salmon sperm. 5 µL of extracted unknown or control DNA was added to each well of a 96 well plate (Applied Biosystems), and the optical lid was closed. The reaction was performed in the ABI Prism 7700 (Applied Biostsystems). The thermal cycling conditions used were 50° C for 2 min, 95° C for 10 minutes, then 60 cycles of 95° C for 15 seconds and 60° C for 30 seconds.

DNA amplification was determined by the detection of fluorescent emission between 500 and 660 nm from the FAM (6-carboxy-fluorescein) reporter dye from the TaqMan probe as it is freed from the quencher, TAMRA (6-carboy-tetramethyl-rhodamine), by the action of the 5'-3' nuclease activity of the Taq polymerase. The fluorescent intensity versus the cycle number of a typical reaction is shown in Figure 3-1. The number of DNA copies of gB present in the sample is proportional to the cycle number at which the fluorescent emission reaches the threshold level. The standard curve was derived from the average of each of the triplicate control samples, Figure 3-2. The curve was accepted if the slope was within the range -3.0 to -3.8 and the regression coefficient of the curve (r^2) was 0.98 or greater. The DNA copy number of the unknown samples was then interpolated from the standard curve. In order to determine the copy numbers per ml of blood, the duplicate results were averaged, and multiplied by 200. The lower limit of detection was 200 copies per ml. Samples prior to 1/7/02 were quantified retrospectively from frozen DNA extracts, and samples obtained after 1/7/02 were quantified prospectively.

Figure 3-1 Real Time PCR fluorescence emission plot for serially diluted control samples.

The log fluorescence emission versus cycle number plot of serially diluted control samples. Each control sample contains a known copy number of gB plasmids, and is analysed in triplicate on the ABI Prism 7700, using a TaqMan probe.

Figure 3-2 Standard curve for determination of HCMV viral load.

A plot of the standard curve derived from serially diluted samples with known copy numbers of gB. This plot is used to determine the HCMV viral load of DNA extracts of whole blood by interpolation. The correlation coefficient in this example is 0.982 and the slope is -3.471.

3.2.4 Calculation of HCMV Replication Kinetics

The initial viral replication rate (K_{max}, day^{-1}) was calculated as the difference between the natural log of the first positive HCMV PCR result and the previous negative result (assigned a value of 200 copies/ml as this is the lower limit of detection of the assay), or between the natural log of the first two HCMV PCR positive results, whichever was the greater, divided by the number of days between samples.

The "mean" viral replication rate (K_{mean}, day^{-1}) was determined from the slope of a line fitted by linear regression to the viral load in log_{10} genome copies/ml versus days post transplantation up to the peak viral load and prior to initiation of therapy. The result was raised to the power of 10 and converted to the natural log.

The decline rate ($K_{decline}$, day^{-1}) was determined from the slope of the linear regression line fitted to the viral load on the linear part of the downward curve of the viral load in log_{10} genome copies/ml versus days post transplant. The result was raised to the power of 10 and converted to the natural log.

3.3 Results

3.3.1 Patient outcomes

Between 1/1/01 and 31/12/04, 57 allogeneic stem cell transplant recipients with at least one episode of HCMV infection based on two consecutive positive PCR results were identified. The characteristics of this group of patients are shown in Table 3-1.

The median time to first HCMV infection following transplant was 37 days, ranging from -7 to 474 days post transplant. Two patients experienced HCMV infection during conditioning therapy but prior to the infusion of donor stem cells, and one patient experienced late HCMV infection post transplant, after immunosuppressive treatment for GVHD following a donor lymphocyte infusion. The median total duration of viraemia was 17 days, with a range of 4 to 59 days, while three patients failed to clear the virus prior to death due to HCMV disease.

Forty-nine patients received HCMV specific antiviral therapy prior to clearance of viraemia, with 24 patients initiated on ganciclovir monotherapy, 22 patients on a combination of ganciclovir and foscarnet, one patient on valganciclovir and two patients on foscarnet alone. In addition six patients were HCMV PCR negative at the time of initiation of antiviral therapy, and two patients successfully cleared the virus from their blood spontaneously and were not initiated on antiviral therapy.

Among the patients receiving HCMV specific antiviral therapy, 13 patients had a change in drug therapy. Ten patients were changed from ganciclovir monotherapy, six to combination therapy with foscarnet and ganciclovir, three to foscarnet monotherapy and one to valganciclovir monotherapy. In six patients this was because of persistent viraemia, in two because of ganciclovir induced pancytopaenia, and two patients to facilitate outpatient therapy. The median time for change from ganciclovir therapy was 12 days (range 5 to 21 days). Two patients were changed from combination ganciclovir and foscarnet therapy to ganciclovir monotherapy due to foscarnet induced side effects, and one patient from

valganciclovir to foscarnet, because of persistent viraemia. The median time to viral clearance following initiation of therapy was 14 days (mean 15.3 days, range 2 to 39 days) although an additional three patients failed to clear viraemia prior to death.

Seventeen patients (31%) experienced a second HCMV infection, with a medium time following first infection of 17 days (Range 4 to 119 days). Eight patients experienced HCMV disease, two patient experienced HCMV colitis/gastritis, four patients experienced proven HCMV pneumonitis with an HCMV positive BAL, and two patients experienced fatal pneumonitis during HCMV viraemia with a clinical picture consistent with HCMV pneumonitis, onset of HCMV viraemia immediately prior to symptoms but unsuccessful attempt at BAL and persistent viraemia until death. Three patients recovered and five patients experienced a fatal outcome, all due to HCMV pneumonitis. The clinical details of patients with HCMV disease are shown in Table 3-2.

Table 3-1 Clinical characteristics of 57 allogeneic stem cell transplant recipients experiencing HCMV infection between 1/1/01 and 31/12/04.

Number		57
Median Age yrs		29
(range)		(3 - 63)
Gender	Male	23
	Female	34
Underlying Diagnosis	ALL	7
	AML	15
	AA	6
	CML	5
	NHL	6
	Hodgkin's Disease	4
	Other Malignant	5
	Beta Thal	6
	Other Non Malignant	3
Conditioning Regimen	Myeloablative	36
	Non-Myeloablative	21
Campath In Vivo Use	In Vivo Campath	44
	No In Vivo Campath	13
Campath Ex Vivo Use	Yes	16
	No	41
Donor Source	HLA Identical Sibling	37
	Non identical Related	3
	Matched Unrelated	11
	Mismatched Unrelated	6
Stem Cell Source	BM	13
	PBSC	44
GVHD Prophylaxis	None	11
	Tacrilimus	1
	Ciclosporin	16
	Methotrexate and Ciclosporin	30
HCMV Serostatus (Recipient/Donor)	Negative/Positive	2
	Positive/Negative	11
	Positive/Positive	44

ALL, acute lymphoblastic leukaemia; AML acute myeloid leukaemia; AA aplastic anaemia; CML, chronic myeloid leukaemia; NHL, non-Hodgkin's lymphoma; Beta Thal, beta thalassaemia; BM, bone marrow; PBSC, peripheral blood stem cell

Table-3-2 Clinical characteristics of patients with HCMV viraemia and disease.

	Age	*Sex*	*HCMV Serology (R/D)*	*Donor Source*	*Stem Cell Source*	*Conditioning Regimen*	*Campath In Vivo*	*Campath Ex Vivo*	*GVHD Prophylaxis*	*Steroid Use*	*Site of Disease*	*Outcome*
Beta Thal	18	Male	Pos/Pos	HLA identical sibling	PBSC	Flu, Cyclo, Busulfan	Yes	No	MTX and CsA	No	Lung	Fatal
AA	29	Male	Pos/Pos	HLA identical sibling	BMT	Flu, Cyclo	Yes	No	MTX and CsA	No	Lung	Fatal
CML	22	Male	Pos/Pos	Non-identical sibling	PBSC	TBI, Flu, cyclo	Yes	Yes	None	No	Lung	Fatal
Hodgkin's Disease	18	Male	Pos/Pos	Non-identical sibling	PBSC	Flu, Cyclo	Yes	Yes	CsA	No	Lung	Survived
NHL	36	Male	Pos/Pos	Mismatch Unrelated	PBSC	Flu, BEAM	Yes	No	MTX and CsA	Yes	Lung	Fatal
NHL	55	Male	Pos/Pos	Mismatch Unrelated	PBSC	Flu, BEAM	Yes	No	MTX and CsA	Yes	Colon	Survived
NHL	14	Female	Pos/Neg	HLA identical sibling	BM	Cyclo, TBI	Yes	No	CsA	Yes	Lung	Fatal
NHL	55	Male	Pos/Neg	Mismatch Unrelated	PBSC	Flu, Mel	Yes	No	CsA	Yes	Gastric Retina	Survived

Beta Thal, beta thalassaemia; AA, aplastic anaemia; CML, chronic myeloid leukaemia; NHL, non-Hodgkin's lymphoma; PBSC, peripheral blood stem cell; BM, bone marrow; Flu, fludarabine; Cyclo, cyclophosphamide; TBI, total body irradiation; BEAM, carmustine, etoposide, cytarabine; melphalan; Mel, melphalan;

3.3.2 Quantitative HCMV PCR Results

The median initial HCMV viral load was 3.0 $\log_{10}$ genome copies/ml (range 2.4 to 4.3 $\log_{10}$ genome copies/ml). The distribution is shown in Figure 3-3. The median peak HCMV viral load during the first HCMV viraemic episode was 3.8 $\log_{10}$ genome copies/ml (range of 2.4 to 5.3 $\log_{10}$ genome copies/ml, Figure 3-4). In 12 cases, the peak viral load was the initial viral load. The median frequency of sampling during viraemia was 2 samples per week, with a range of 1 to 4.5 samples per week.

Figure 3-3 Frequency histogram of the initial HCMV viral load.

A frequency histogram of the initial $\log_{10}$ genome copies/ml HCMV viral load from the first 57 allogeneic stem cell transplant recipients experiencing HCMV infection is shown. The median initial viral load was 3.0 $\log_{10}$ genome copies/ml (range 2.4 to 4.3 $\log_{10}$ genome copies/ml).

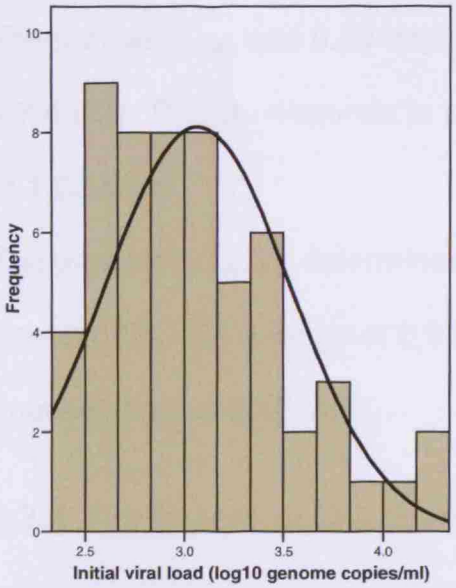
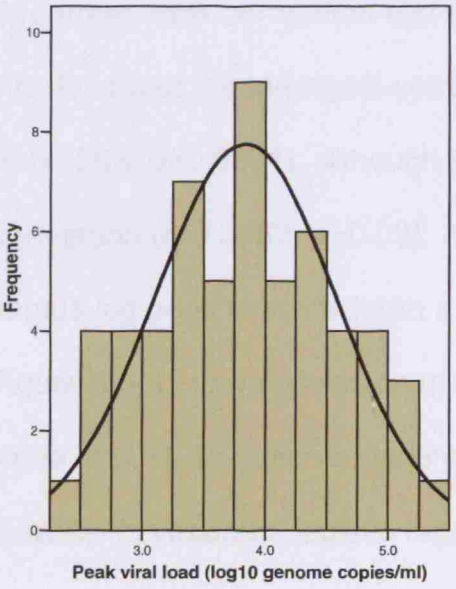


Figure 3-4 Frequency histogram of the peak HCMV viral load.

A frequency histogram of the peak $\log_{10}$ genome copies/ml HCMV viral load from 57 allogeneic stem cell transplant recipients experiencing HCMV infection is shown. The median peak viral load was 3.8 $\log_{10}$ genome copies/ml (range 2.4 to 5.3 $\log_{10}$ genome copies/ml)



3.3.3 HCMV Replication Kinetics

The median K_{max} was 0.38 day^{-1} (mean 0.4505, with a range of 0.05 to 1.7 day^{-1}). This corresponds to a median initial HCMV viral doubling time of 1.82 days.

The median K_{mean} as determined by linear regression was 0.32 day^{-1} (mean 0.35, with a range of 0.05 to 0.9 day^{-1}), corresponding to an HCMV doubling time of 2.17 days.

3.3.4 Predictors of Duration of Viraemia

The relationship between viral load dynamics and total duration of therapy was examined. To assess if there was a significant correlation between the total duration of viraemia and viral load parameters a Spearman rank correlation test was performed, Table 3-3. The log peak viral load had the strongest correlation with the total duration of viraemia ($r=0.7953$, $p<0.0001$), although the K_{max} also shows a significant correlation ($r=0.2853$, $p=0.03$). A scatter plot of the duration of viraemia versus log peak viral load with a linear regression line fitted is shown in Figure 3-5. Linear regression using the least squares method gave an r^2 value of 0.54. In order to identify the main factors determining the duration of viraemia, logistic regression analysis with a binary outcome of total duration of viraemia less than or greater than 14 days was performed. Continuous variables entered into the model were log peak viral load, log initial viral load, K_{max} and K_{mean}. Categorical variables entered into the model were an acute GVHD score greater than I, in vivo

use of Campath-1H and steroid use during viraemia. The results of univariable analysis are shown in Table 3-4. The results show that log peak viral load, log initial viral load and use of Campath-1H in vivo for conditioning predict the duration of viraemia. Using a multivariable model, with backwards conditional selection and a probability criteria for entry into the model of 0.05, and for removal of 0.1, both log peak viral load with a RH of 7.5 per quarter log increase in peak viral load (95% CI 1.7 to 32.5, p = 0.007) and K_{mean} viral replication rate with a RH of 0.38 per 0.1 day^{-1} (95% CI 0.15 to 0.99, p = 0.047) remain significant, although in vivo Campath-1H use is of borderline significance, RH 20.6 (95% CI 0.9 to 474.3, p=0.06).

Table-3-3 Correlation of initial viral load, peak viral load, K_{max} and K_{mean} with total duration of HCMV viraemia in days.

The duration of viraemia for patients not clearing virus prior to death was defined as the difference between the date of the last HCMV PCR positive result prior to death and the first positive result.

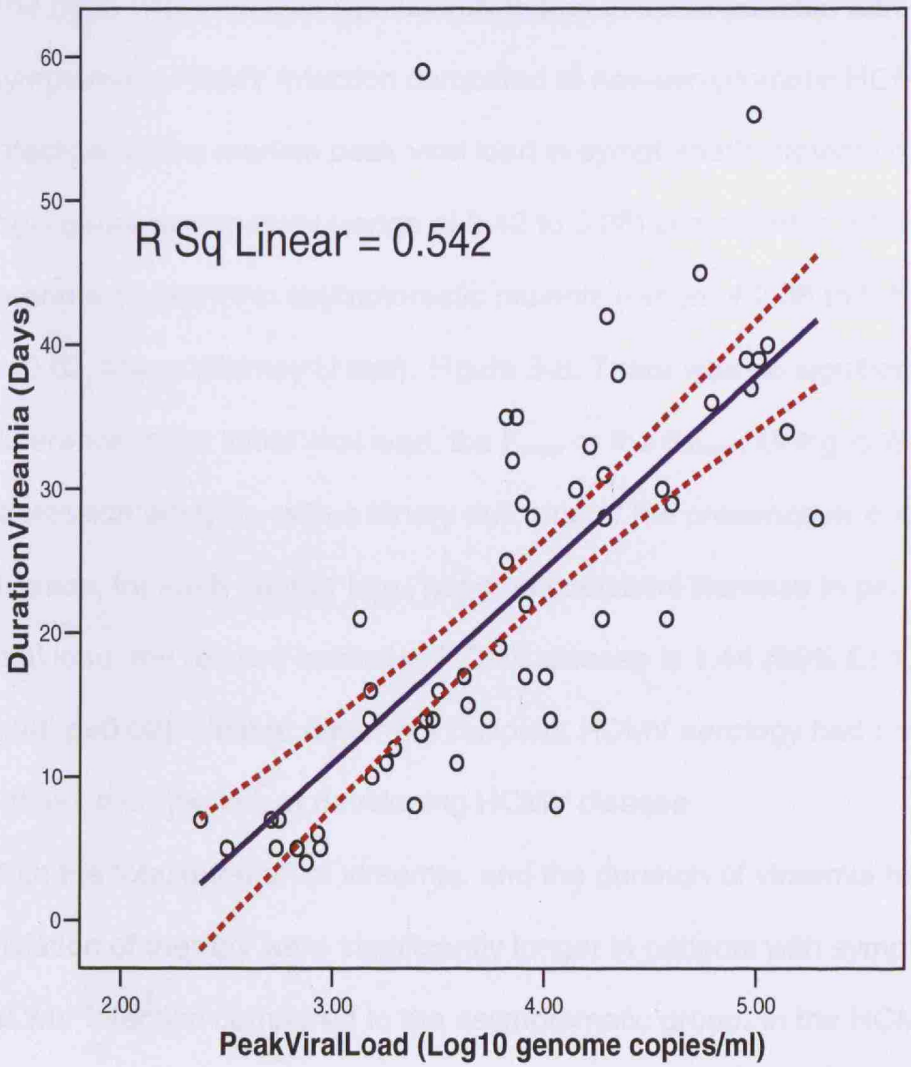
	Initial viral load *log_{10} genome copies/ml*	***Peak viral load*** *log_{10} genome copies/ml*	***K_{max}*** *day^{-1}*	***K_{mean}*** *day^{-1}*
Number	57	57	57	57
Spearman rank correlation	0.16	0.79	0.29	0.10
95% confidence interval	-0.11 to 0.41	0.67 to 0.88	0.02 to 0.51	-0.08 to 0.43
p value (two-tailed)	0.24	P<0.0001	0.03	0.16

Table-3-4 Univariable logistic regression analyses of factors predicting a duration of viraemia of 14 days or more.

Variable	*Relative Hazard*	*95% CI*	*Significance (P value)*
Peak Viral Load (each quarter log_{10} genome copies/ml)	3.16	1.67-5.97	<0.0001
Initial Viral Load (each quarter log_{10} genome copies/ml)	1.55	1.03-2.33	0.03
K_{max} (each 0.1 per day increase in viral replication rate)	1.26	0.5-1.68	0.1
K_{mean} (each 0.1 per day increase in viral replication rate)	1.1	0.83-1.46	0.52
Acute GVHD > I	0.97	0.25-3.69	0.96
Campath-1H in vivo	4.54	1.22-16.88	0.02
Steroid Use	0.69	0.21-2.32	0.55

Figure 3-5 Duration of viraemia versus peak viral load

A scatter plot of the duration of viraemia in days versus peak viral load (log_{10}genome copies/ml) is shown. A linear regression line is fitted and has a slope of 13.5 (95% CI 10.14 – 16.85), with an r^2 of 0.542 ($p<0.0001$). The linear regression line is plotted in blue, with the 95% confidence interval plotted as dashed red lines.



3.3.5 Predictors of HCMV Disease

The peak viral load was significantly higher in those patients with symptomatic HCMV infection compared to non-symptomatic HCMV infection, with a median peak viral load in symptomatic patients of 4.45 log_{10} genome copies/ml (range of 3.42 to 5.28) compared to 3.82 log_{10} genome copies/ml in asymptomatic patients (range of 2.38 to 5.14, p=0.02, Mann Whitney U test), Figure 3-6. There was no significant difference in the initial viral load, the K_{max} or the K_{mean}. Using logistic regression analysis, with a binary outcome of the presence or absence of disease, for each quarter log_{10} genome copies/ml increase in peak HCMV viral load, the relative hazard of HCMV disease is 1.44 (95% CI 1.05 to 1.96, p=0.02). Neither donor nor recipient HCMV serology had an influence on the risk of developing HCMV disease.

Both the total duration of viraemia, and the duration of viraemia following initiation of therapy were significantly longer in patients with symptomatic HCMV infection compared to the asymptomatic group. In the HCMV symptomatic group the median duration of viraemia was 32 days and the median duration of viraemia following initiation of therapy was 20 days, while in the asymptomatic group the median duration of viraemia was 16 days and the duration of viraemia following therapy was 13 days (p values 0.003 and 0.04 respectively, Mann Whitney U test), Figure 3-7 and Figure 3-8. The duration of viraemia in the HCMV symptomatic group is likely to be an underestimate as the duration of viraemia in the three patients failing to clear viraemia prior to death was defined as up to the

last viral load before death. Neither the time to initiate antiviral therapy (median of 6 days in the asymptomatic group and 7 days in the symptomatic group, p=0.40 Mann Whitney U test) or the $K_{decline}$ rate (median of 0.37 day^{-1} in the asymptomatic group and 0.25 day^{-1} in the symptomatic group, p=0.099) was significantly different between the two groups.

If the log peak viral load, total duration of viraemia, K_{mean} and $K_{decline}$ are entered into a multivariable model, with a binary outcome of the presence or absence of disease, then only the total duration of viraemia remains a statistically significant risk factor for symptomatic HCMV infection, with a RH of 1.12 per day of viraemia (95% confidence interval 1.04 to 1.22, p=0.006).

Figure-3-6 Peak viral load according to the presence or absence of HCMV disease.

A scatter plot of the peak viral load (log_{10}genome copies/ml) is plotted for patients with or without HCMV disease. The horizontal lines represent the median and the interquartile range. The median log peak viral load of patients with disease is 4.4 log_{10} genome copies/ml, and for patients without disease is 3.7 log_{10} genome copies/ml. The difference is statistically significant (p=0.02 Mann Whitney U test).

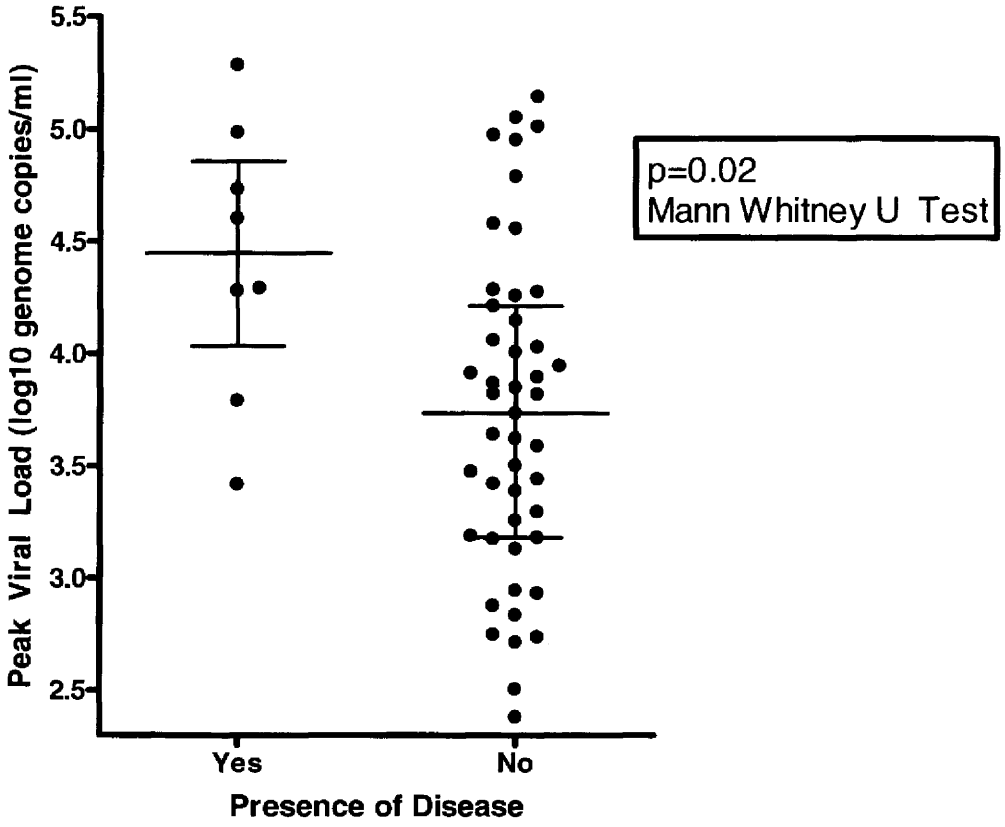


Figure 3-7 Duration of viraemia according to the presence or absence of HCMV disease.

A scatter plot of the duration of viraemia for patients with or without HCMV disease is plotted. Duration of viraemia in patients not clearing virus was calculated to the last HCMV PCR result, therefore giving an underestimate of likely duration of viraemia. The horizontal lines represent the median value and the interquartile range. The median duration of viraemia in patients with disease is 32 days, and in patients without disease is 16 days. This difference is statistically significant (p=0.004, Mann Whitney U test).

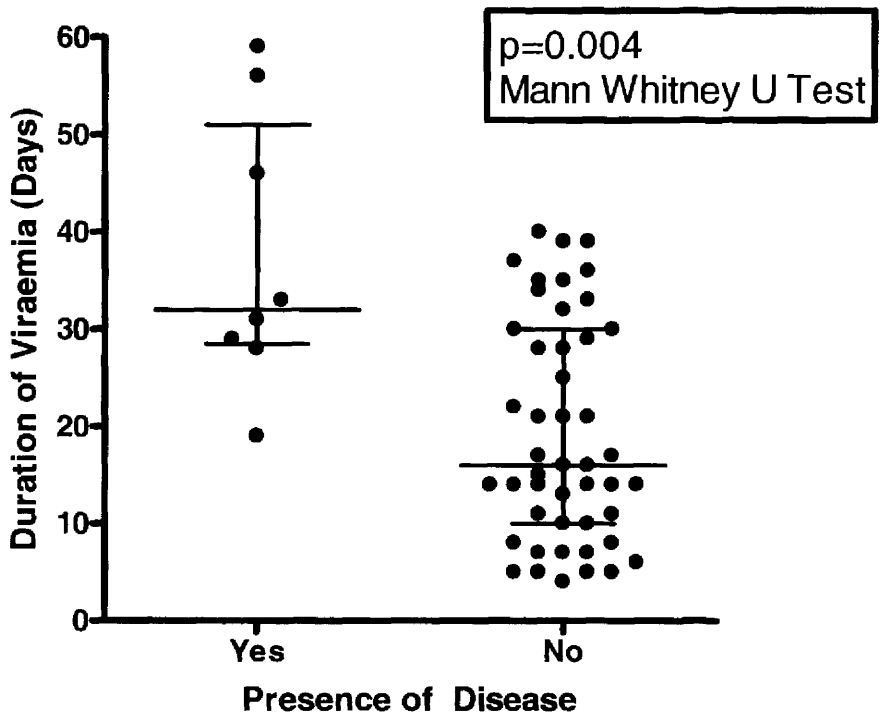
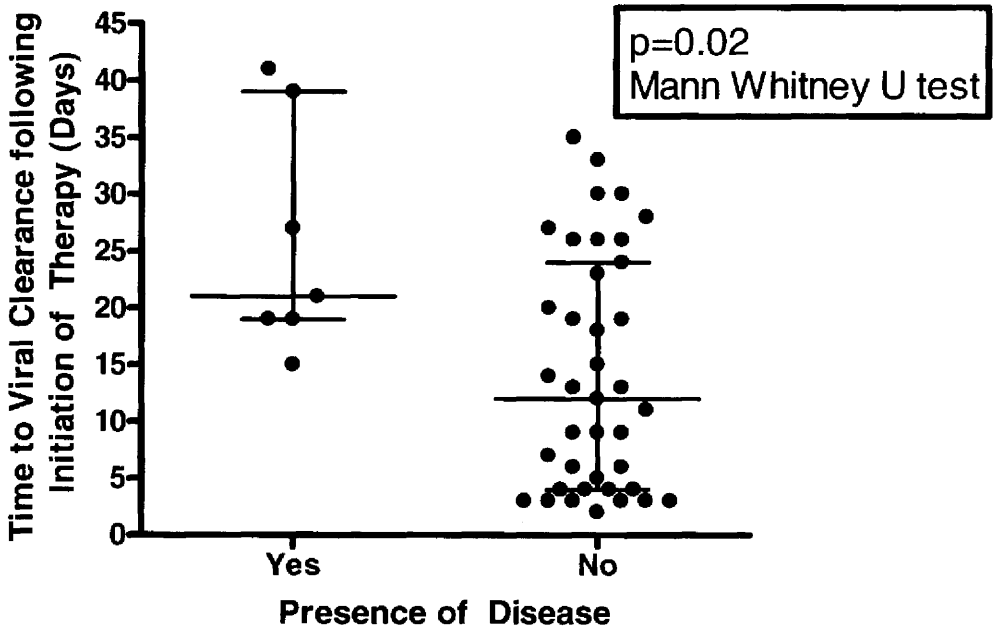


Figure 3-8 Time to viral clearance following initiation of therapy according to the presence or absence of HCMV disease.

A scatter plot of the time to viral clearance in days following initiation of therapy is plotted according to the presence or absence of HCMV disease. The horizontal lines represent the median value and the interquartile range. The median number of days to clear viraemia in patients experiencing HCMV disease is 21 days, and in patients not experiencing disease is 12 days. This difference is statistically significant (P=0.02, Mann Whitney U test).



3.3.6 Viral Decay Curves and Response to Therapy

The viral decline curves were evaluated in 57 patients. Two patients did not receive antiviral therapy but were able to clear the virus spontaneously. Both patients only had two positive HCMV PCR results. Another 6 patients cleared viraemia before or at the time antiviral therapy was initiated. Among the remaining 49 patients, the group was divided into those patients in whom the viral load continued to rise following initiation of therapy, defined as the peak viral load occurring greater than two days after therapy (n= 21) and those patients in whom the viral load responded immediately to therapy, with the peak viral load less than or equal to two days following initiation of therapy (n= 28). Thus, based on an intention to treat analysis, 38 percent (21 out of 55) of patients experienced a peak viral load after the initiation of therapy, reflecting an increasing level of viraemia despite the initiation of therapy. In order to identify the risk factors for a delayed peak viral load, a comparison of the potential contributing factors known for each group at the time antiviral therapy was initiated was performed, using the Fisher exact test for categorical factors, and the Mann Whitney U (2 tailed) test for continuous variables, Table 3.5. A scatter plot of the factors found to be significantly different between the two groups is shown in Figure 3-9. A higher K_{max}, higher K_{mean} and a higher baseline viral load at initiation of therapy were all predictive of a delayed peak viral load after initiation of therapy, while an acute GVHD score of 2 or more was of borderline significance ($p=0.06$). The type of treatment initiated did not influence the likelihood of

response. Not surprisingly, patients that experienced a delayed rise in viral load after initiation of therapy are also significantly more likely to experience symptomatic disease. When all the factors are entered into a multivariable logistic regression model with a binary outcome of a delayed peak viral load after initiation of therapy, using a backwards conditional selection criteria, only the K_{mean} (mean replication rate prior to therapy) remained an independently significant factor in predicting response to therapy, Table 3-6. It was possible to calculate the decay curves in the presence and absence of HCMV specific therapy in all but two patients (one due to viral load increasing at the time of death and the second due to the inability to fit a linear regression curve). The decline rate in the absence of therapy was calculated in eight patients who either did not receive therapy (n=2) or in whom viraemia cleared prior to initiation of therapy (n=6), with a $K_{decline}$ of 0.25 day^{-1} (range 0.09 to 0.38 day^{-1}), equivalent to a half-life of 2.77 days. A further 41 patients had a decline only in response to therapy, with a median $K_{decline}$ of 0.41 day^{-1} (range 0.14 to 1.48 day^{-1}), equivalent to a half-life of 0.69 days. This difference in the viral decay rates was significant (Mann Whitney U Test p=0.02, Figure 3-10). In six cases, where therapy was initiated after the viral load had started to decline, but before viral load was undetectable it was possible to compare the decline rates pre and post therapy, Figure 3-11. This showed an increase in the $K_{decline}$ in 5 out of the 6 patients following initiation of therapy, with an average 4 fold increase in the rate of decline (mean of 0.19 day^{-1} prior to treatment vs. mean of 0.74 day^{-1} following initiation of treatment, p=0.02, Paired T Test).

Table-3-5 Characteristics of immediate responders versus delayed responders to HCMV specific therapy.

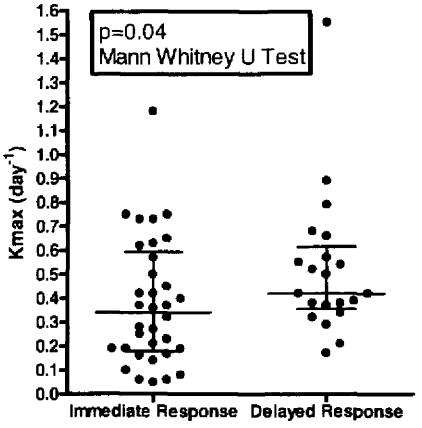
Factor	*Immediate Response to Therapy*	*Delayed Response To Therapy*	*Significance (p value)*
Number	34	21	
Median age in yrs (range)	30 (10-63)	29 (3, 55)	0.64
In Vivo Campath-1H Use (%)	24 (70.6%)	19 (90.5%)	0.10
GVHD score > I (%)	5 (14.7%)	8 (38.1%)	0.06
Steroid use (%)	9 (26.5%)	8 (38.1%)	0.39
Treatment			
Ganciclovir	16 (47%)	10 (47.6%)	0.53
Foscarnet/Ganciclovir	14 (41%)	10 (47.6%)	0.53
Foscarnet	3 (8.8%)	0	-
Valganciclovir	1 (2.9%)	1 (4.8%)	-
Median K_{max} day^{-1} (range)	0.34 (0.05-1.18)	0.42 (0.17-1.55)	0.04
Median K_{mean} day^{-1} (range)	0.25 (0.05-0.75)	0.39 (0.18-0.90)	0.02
Median initial viral load log_{10} genome copies/ml (range)	2.98 (2.38-4.3)	3.04 (2.48-4.33)	0.47
Median baseline viral load log_{10} genome copies/ml (range)	3.26 (2.30-5.07)	3.69 (2.41-5.14)	0.05
Median time to initiate therapy from 1st PCR+ days (range)	7 (1-38)	6 (2-14)	0.44
Symptomatic Infection (%)	2 (5.9%)	6 (28.6%)	0.04

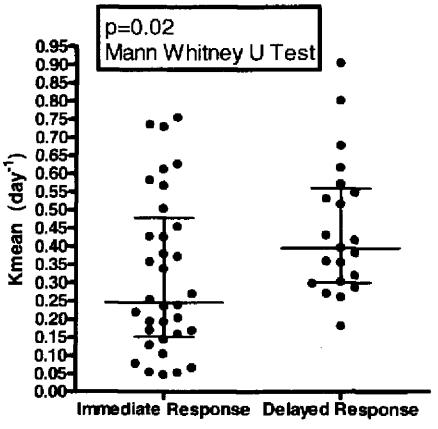
Table-3-6 Multivariable logistic regression analysis of the risk factors for delayed response to therapy.

Factor	*Relative Hazard*	*95% CI*	*Significance (p value)*
GVHD score > I	4.0	0.95–16.6	0.08
In vivo Campath-1H	5.6	0.84-37.6	0.06
K_{mean} (per 0.1 increase day^{-1})	1.4	1.02-1.87	0.004

Figure 3-9 K_{max}, K_{mean}, and baseline viral load according to immediate or delayed response to HCMV therapy.

Scatter plots of the difference in K_{max}, K_{mean} and baseline viral load (log_{10} genome copies/ml) at initiation of therapy between immediate responders (n=34) and delayed responders (n=21) to antiviral therapy are plotted. The horizontal lines indicate the median value and the interquartile range. The median K_{max} in immediate responders is 0.34 day^{-1} and in delayed responders is 0.42 day^{-1} (p=0.04 Mann Whitney U Test). The median K_{mean} in immediate responders is 0.25 day^{-1} and in delayed responders is 0.39 day^{-1} (p=0.02, Mann Whitney U Test). The median baseline viral load in immediate responders is 3.26 log_{10} genome copies/ml and the delayed responders is 3.69 log_{10} genome copies/ml (p=0.05, Mann Whitney U Test).





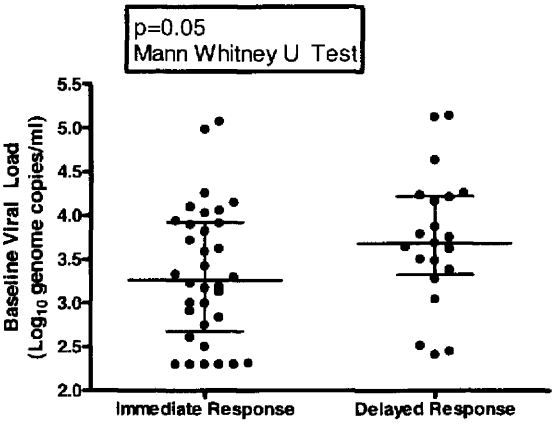


Figure 3-10 Rate of decline in HCMV viraemia according to the absence or presence of antiviral therapy.

A scatter plot of the rate of decline in HCMV viraemia, determined by linear regression of the decay curve, is plotted according to the absence (8 patients) or presence (41 patients) of antiviral therapy. The horizontal lines represent the median value and the interquartile range. The median rate of decline in the absence of therapy is 0.25 day^{-1}, and the median rate of decline in the presence of antiviral therapy is 0.41 day^{-1}. The difference is statistically significant (p=0.02, Mann Whitney U Test).

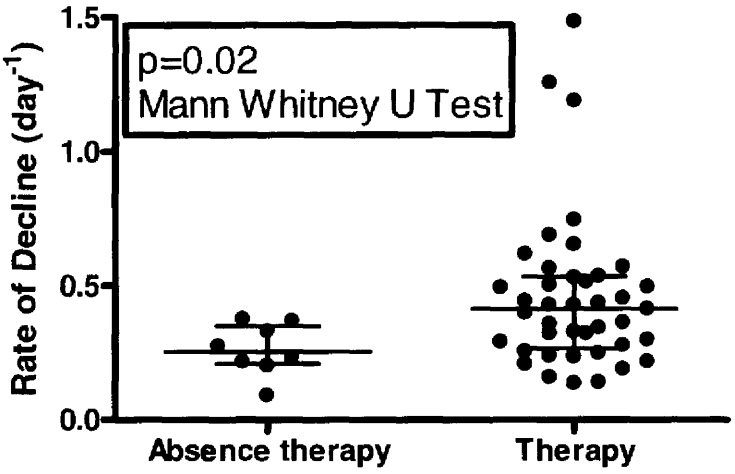
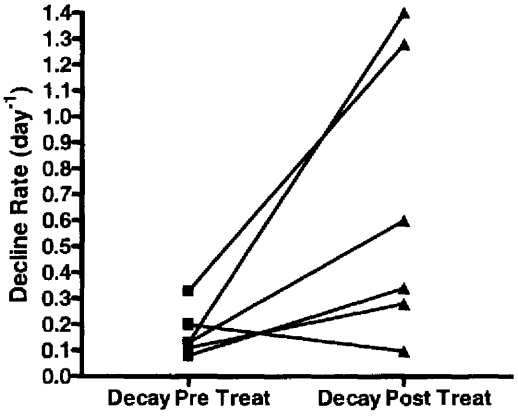


Figure-3-11 HCMV decline rates before and after initiation of antiviral therapy.

A comparison of the HCMV decline rates in six patients in whom the effect of therapy on the decline rate could be calculated before and after initiation of antiviral therapy is shown. The mean rate of decline before treatment was 0.19 day^{-1}, and after initiation of treatment was 0.74 day^{-1}. The difference is statistically significant (p=0.02, Paired T Test).



3.3.7 Comparison of Ganciclovir and Combination Ganciclovir/Foscarnet for the Treatment of HCMV Viraemia

Using viral load dynamics it was possible to compare the efficacy of HCMV specific antiviral therapy at clearing viraemia. Based on an intention to treat analysis, there was no difference in the rate of viral clearance. However, six patients receiving ganciclovir monotherapy were changed to combination therapy due to a perceived poor response to monotherapy. As the median time to change therapy (in those patients not clearing the virus before therapy was initiated) was 12 days, and only one patient changed therapy before 7 days, the change in viral load in the first 7 days of therapy was compared, and is show in Figure 3-12. An alternative way of assessing the difference is to use a 'crossover' analysis, whereby patients that had a therapy change were put in the alternative group and the serial decline in viral load and the 95% confidence intervals plotted at four day intervals, Figure 3-13. No difference between ganciclovir and combination ganciclovir/foscarnet therapy was observed, although this was a non-controlled, retrospective study.

Figure 3-12 Comparison of change in viral load at day seven between ganciclovir monotherapy and combination ganciclovir/foscarnet therapy.

A scatter plot comparing the change in log_{10} genome copies/ml viral load at day 7 between patients receiving ganciclovir monotherapy (n=13) and combination ganciclovir/foscarnet therapy (n=20). The horizontal lines indicate the median value and the interquartile range. The median change in HCMV viral load at day 7 for ganciclovir monotherapy is 0.55 log_{10} genome copies/ml, and for combination ganiclovir/foscarnet therapy is 0.66 log_{10} genome copies/ml.

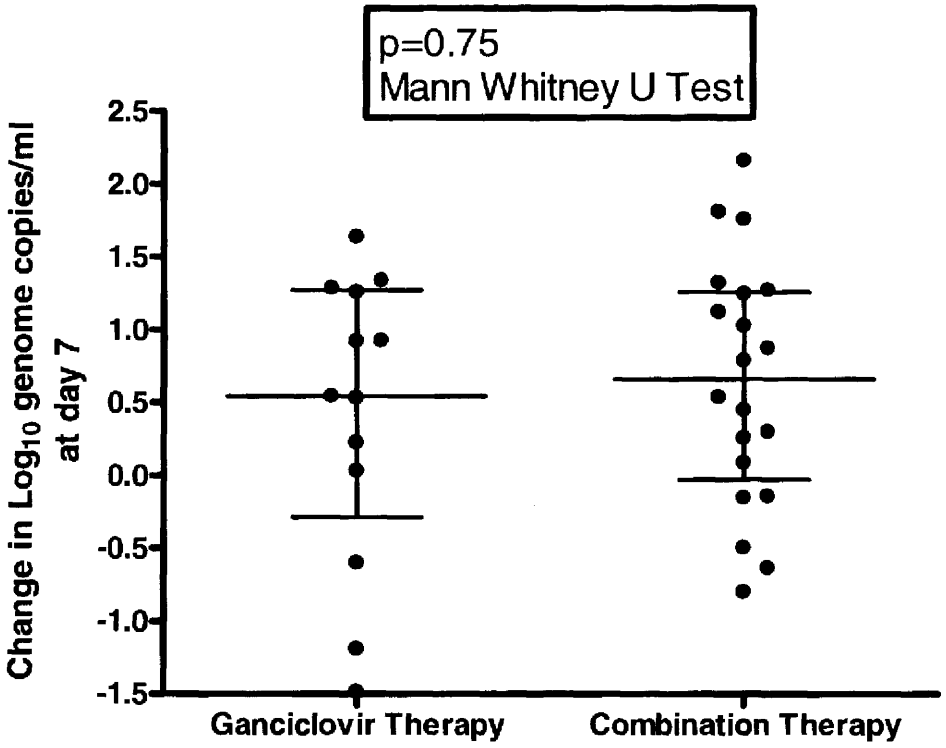
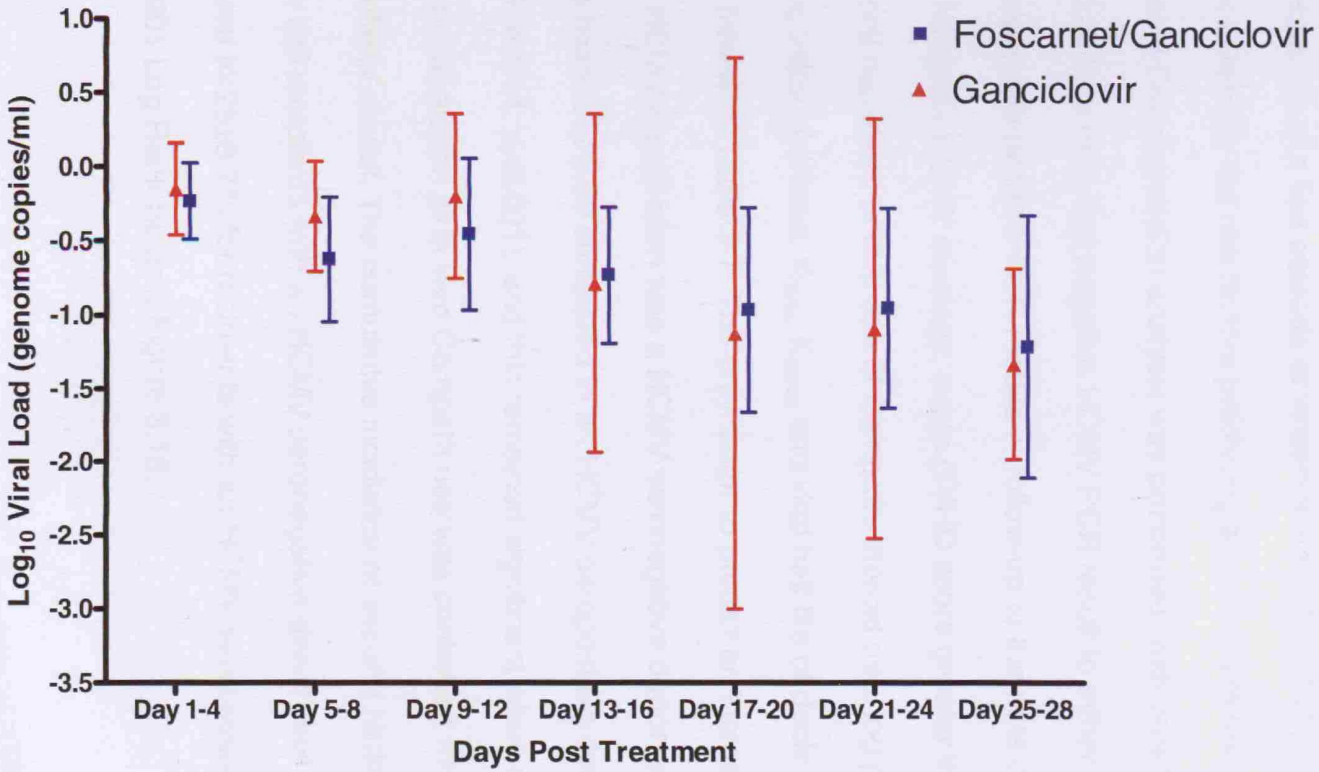


Figure 3-13 Comparison of the change in viral load at four day intervals between ganciclovir monotherapy and combination therapy with ganciclovir/foscarnet.

The change in $\log_{10}$ genome copies/ml viral load following initiation of therapy at four day intervals is shown for ganciclovir monotherapy (red) and foscarnet/ganciclovir combination therapy (blue). The triangle/square symbols indicate the mean values and the error bars the 95% confidence interval. Patients undergoing a change in therapy are crossed over into the other group. There is no difference in response to therapy between ganciclovir monotherapy and combination therapy with ganciclovir/foscarnet.



3.3.8 Predictors of Recurrence of Infection

Seventeen of the 54 patients that cleared the initial HCMV infection experienced a second episode of infection, at a median time of 20 days (range 4 to 119 days) following clearance of the first infection. The Kaplan Meier estimate of the cumulative incidence of a second HCMV infection after clearance of a first episode of viraemia was 37.9±7.8%, Figure 3-14. In order to identify the risk factors predicting a second infection, univariable Cox regression analysis was performed (with time to infection defined as time from first negative HCMV PCR result to either the first of two consecutive positive PCR results or follow-up to the date of last PCR result) for donor HCMV serology, acute GVHD score greater than 1, ex vivo T-cell depletion, in vivo use of Campath, steroid use, log peak viral load, log initial viral load, K_{max}, K_{mean} and viral half life of decline post first HCMV infection, Table 3-7. The only factor to predict an increased risk of further HCMV reactivation was a HCMV seronegative donor, with a relative hazard of 6.59 compared to an HCMV seropositive donor (95% CI 2.49 to 17.4, $p<0.001$), and this remained significant when either ex-vivo T-cell depletion or in vivo Campath use was controlled for in a multivariable model. The cumulative incidence of second HCMV infection in stem cell recipients with an HCMV seronegative donor was 80±12.7%, compared to 28±8.7% for recipients with an HCMV seropositive donor ($p<0.0001$ Log Rank Score), Figure 3.15.

Table 3-7 Univariable Cox regression analysis for the risk factors for second HCMV infection.

Factor	*Relative Hazard*	*95% CI*	*Significance (P value)*
HCMV seronegative donor	6.59	2.49-17.41	<0.001
Acute GVHD score > I	1.16	0.57-3.99	0.80
Ex vivo T-cell depletion	1.50	0.57-3.99	0.41
In vivo Campath-1H Use	1.37	0.39-4.76	0.62
Steroid therapy	1.44	0.55-3.79	0.46
Peak viral load (each quarter $\log_{10}$ genome copies/ml)	0.72	0.35-1.45	0.35
Initial viral load (each quarter $\log_{10}$ genome copies/ml)	0.39	0.11-1.39	0.15
K_{max} (each 0.1 per day increase in viral replication rate)	0.12	0.01-1.51	0.1
K_{mean} (each 0.1 per day increase in viral replication rate)	2.55	0.45-14.50	0.29
Half life of decline (days)	1.03	0.97 – 1.10	0.32

Figure 3-14 Kaplan Meier estimate of the cumulative incidence of second HCMV infection.

The Kaplan Meier estimate of the cumulative incidence if second HCMV infection is plotted in 54 patients who cleared the first episode of HCMV viraemia. Time to infection or follow-up was measured from the date of clearance of first viraemia. The cumulative incidence of second HCMV infection is 37.9±7.8%. The cross bars indicate censored data.

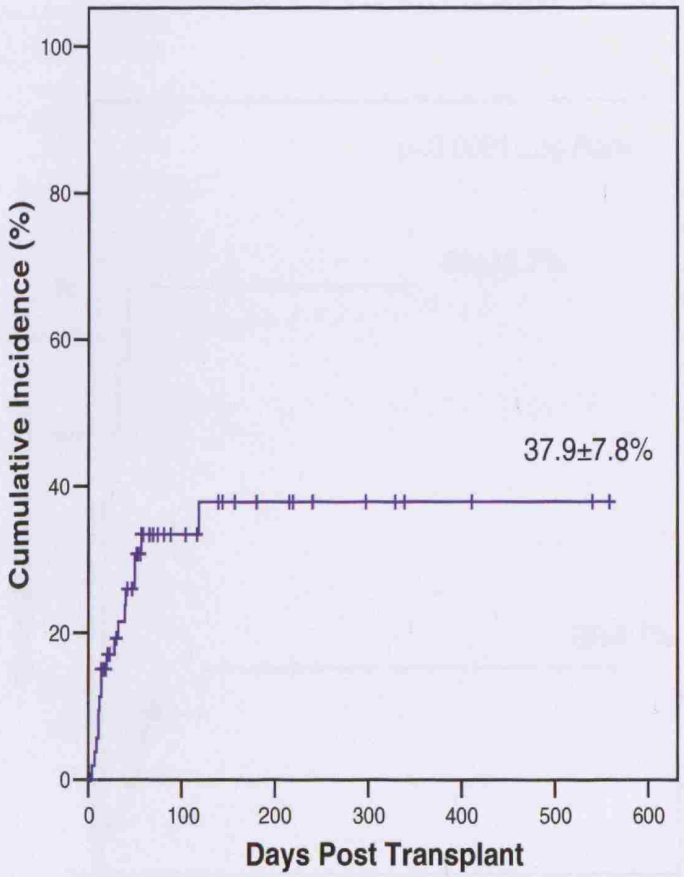
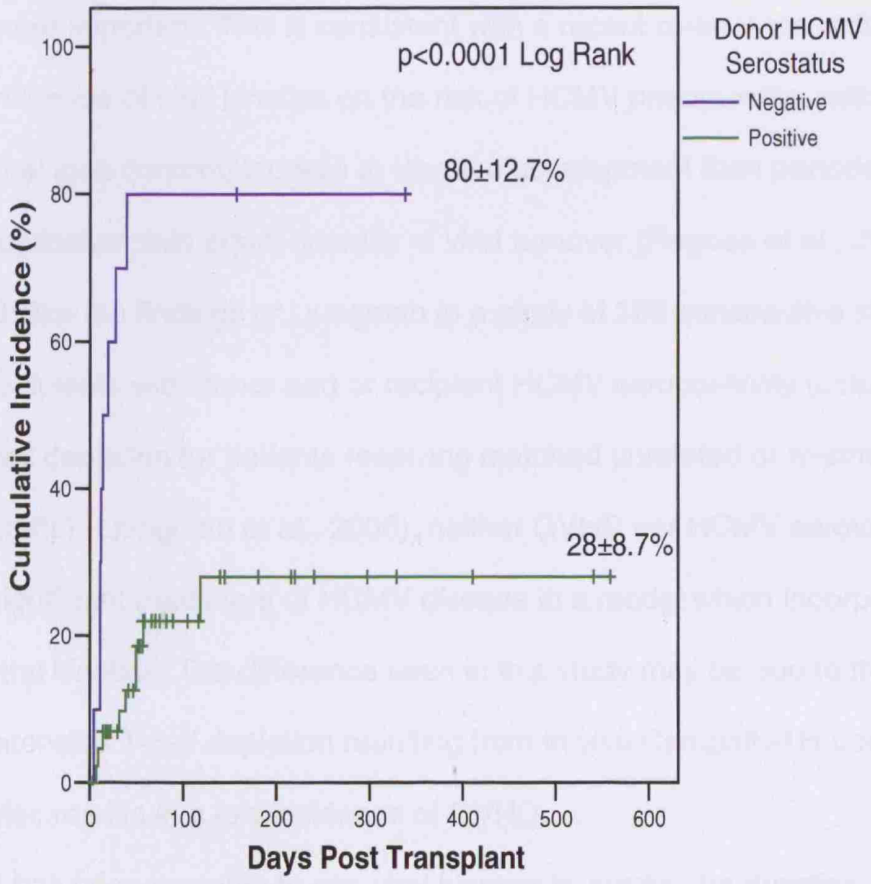


Figure 3-15 Kaplan Meier estimate of the cumulative incidence of second HCMV infection according to donor HCMV serostatus.

The Kaplan Meier estimate of the cumulative incidence of second HCMV infection in 54 patients who cleared the first episode of HCMV viraemia. Time to infection or follow-up was measured from the date of clearance of first viraemia. The cumulative incidence of second HCMV infection in patients with an HCMV seropositive donor (n=44, green line) is 28±8.7%, and in patients with a seronegative donor (n=10, blue line) is 80±12.7%. The difference is statistically significant (p<0.0001, Log Rank Score). The cross bars indicate censored data.



3.4 Conclusions

This study confirms that in the era of pre-emptive therapy, peak viral load still significantly correlates with HCMV disease. However, in symptomatic patients, the duration of viraemia was twice that of non-symptomatic patients and it was the most significant risk factor for disease in a multivariable model, suggesting that the total exposure to viraemia is more important. This is consistent with a recent re-analysis of the influence of viral kinetics on the risk of HCMV pneumonitis, with the peak viral load contributing less to disease development then periods of viral replication with equal quantity of viral turnover (Regoes et al., 2006). Unlike the findings of Ljungman in a study of 162 consecutive stem cell recipients with donor and or recipient HCMV seropositivity (using ATG T-cell depletion for patients receiving matched unrelated or mismatched grafts) (Ljungman et al., 2006), neither GVHD nor HCMV serology were significant predictors of HCMV disease in a model which incorporated viral kinetics. The difference seen in this study may be due to the more intensive T-cell depletion resulting from in vivo Campath-1H use which also results in a low incidence of GVHD.

It has been possible to use viral kinetics to predict the duration of therapy required to clear viraemia, with both higher peak viral loads and faster viral replication rates pre-treatment predicting the need for a longer duration of therapy. Eleven percent (6 out of 55 patients receiving therapy) of patients were initiated on therapy at a time when they were no longer viraemic. Real time viral load kinetics can be used to reduce

unnecessary antiviral therapy. In order to reduce the amount of unnecessary therapy, per-emptive therapy was only initiated when the viral load reached 3000 copies/ml. Verkruyse describes an effective alternative approach of only using ganciclovir (at a dose of 5mg/kg OD) for HCMV viral loads equal or greater than 10000 genome copies/ml loads, although this does risk exposing patients to prolonged low level viraemia (Verkruyse et al., 2006), which has been shown in this study to be a significant risk factor for disease. A smaller Australian study of 46 stem cell transplant recipients identified either a high viral load (>5000 copies/ml) or a persistent viraemia as a risk factor for disease (Howden et al., 2003). Based on the findings of this study, the strategy of initiating antiviral therapy after a single high level (>3000 genome copies/ml) or two or more consecutively PCR positive results at any level is the most appropriate to reduce the risk of HCMV disease. The question of whether the dose of antiviral therapy can be reduced in patients with low level viraemia remains to be evaluated.

In many patients, the viral load was observed to continue rising after initiation of therapy, and this was often interpreted as a failure of antiviral therapy, when in fact it could be predicted from pre-therapy viral kinetics. Gerna has described a similar phenomenon, with viral loads not falling until 5-10 days after initiation of antiviral therapy, and this is more likely to occur in patients receiving steroid therapy for acute GVHD grade II-IV (Gerna et al., 2005). The type of therapy initiated had no influence on a delayed reduction in viral loads. Patients with a delayed reduction in viral loads were at greater risk of HCMV disease, and these patients may be

appropriate for more intensive antiviral therapy or as priorities for adoptive immunotherapy.

Viral kinetics confirmed the equivalence in efficacy of ganciclovir monotherapy and combination ganciclovir/foscarnet therapy, consistent with the findings of Mattes in a randomised trial comparing ganciclovir and foscarnet/ganciclovir combination therapy in both solid organ and stem cell transplant recipients (Mattes et al., 2004). In a large multicentre trial of 213 stem cell transplant patients randomised to either ganciclovir or foscarnet monotherapy, despite equivalence in efficacy, 11% of patients initiated on ganciclovir developed severe neutropaenia (Reusser et al., 2002). Therefore, the choice of antiviral agent should be determined by clinical need (such as avoidance of cytopaenia or presence of renal impairment). The efficacy of valganciclovir compared to ganciclovir to control HCMV infection should also be evaluated using viral load kinetics as has been done for solid organ disease (Mattes et al., 2005).

The strongest risk factor for a second episode of HCMV infection was a seronegative donor, with a cumulative incidence of second HCMV infection of 80 percent, demonstrating the importance of immune regulation of viral replication. However, the rate of decline following first HCMV infection did not influence the risk of second reactivation, unlike Ljungman's findings (Ljungman et al., 2006), or the experience in solid organ transplant, where a second infection is correlated with a slower rate of decline (Humar et al., 2002). The extensive use of Campath-1H in this cohort of patients, due to its T-cell depleting effect and its relatively long

half life (Morris et al., 2003), preventing donor immunity from influencing the first HCMV infection episode, but not later infections may account for these findings. Despite HCMV seropositive patients with seronegative donors being at the greatest risk of recurrent HCMV infections, adoptive immunotherapy is currently only available for recipients of HCMV seropositive donors. Ideally, an HCMV seropositive recipient should receive a graft from a seropositive donor, if such a choice is available. Alternatively, clinical trials evaluating donor vaccination for HCMV should be carried out.

Chapter 4 HCMV Specific Immune Recovery following Allogeneic Stem Cell Transplantation

4.1 Introduction

The role of cytotoxic T lymphocytes in protecting allogeneic stem cell transplant patients from HCMV disease has been studied by Reusser who found that in the absence of ganciclovir prophylaxis, patients with a detectable CTL response by three months did not develop HCMV pneumonia (Reusser et al., 1991). The use of tetramer technology for HCMV specific CD8+ T cells has now been widely applied in transplant patients and has shown the importance of pp65 specific CD8+ T cells in controlling HCMV infection following allogeneic transplantation (Aubert et al., 2001; Cwynarski et al., 2001; Gratama et al., 2001), with a level of two or more HCMV specific tetramer positive cells/μl being indicative of protection against HCMV disease in allogeneic stem cell transplant recipients. However, tetramer technology does not predict the functional capacity of CD8+ T cells, such as their ability to secrete interferon gamma in response to antigenic stimulation (Villacres et al., 2003). Early studies of HCMV specific CD4+ T cell helper function following stem cell transplantation, based on lymphoproliferation assays using ^{3}H thymidine uptake, have demonstrated that recovery of CD8+ CTL function is dependent on CD4+ T cell recovery (Li et al., 1994). Walters demonstrated that following adoptive immunotherapy for HCMV following transplant, persistence of HCMV specific CD8+ CTLs were dependent on the presence of HCMV specific CD4+ T cells (Walter et al., 1995). However lymphoproliferation responses are a function of both the

frequency and the activation status of the responding T cell. Although tetramers can directly enumerate the number of antigen specific cells present (for a known epitope), because of the difficulty in producing MHC class II complexes, due to the difficulty in refolding polymorphic α and β chains (Ferlin et al., 2000) as well as the low frequency of antigen specific CD4+ T cells, only a limited number of Class II tetramers have been successfully produced, such as for Borrelia, influenza and hepatitis C (Day et al., 2003; Lucas et al., 2004; Meyer et al., 2000). HCMV specific class II tetramers have now been described, such as the HLA DR3 tetramer (Bronke et al., 2005), but unlike Class I tetramers, they remain relatively few. Therefore, alternative methods of monitoring HCMV specific CD4+ T cell responses that incorporate an assessment of their functional capacity have been developed. Utilising Brefeldin A, a fungal metabolite which disrupts the golgi apparatus and blocks export of newly produced proteins, following ex vivo antigen stimulation of T cells, it is possible to enumerate the cytokine response by enzyme linked immunospot (ELISPOT) assay or flow cytometry using intracellular cytokine staining techniques (Waldrop et al., 1997). Flow cytometry has the advantage of directly characterising the immunophenotype of the responding cell by the use of additional monoclonal antibodies and selective gating strategies. Up to 1-2 percent of the total CD4+ T cell population of normal HCMV seropositive individuals can be identified as having a T_H1 response to HCMV (Waldrop et al., 1998; Sester et al., 2002). In HIV even higher frequencies of up to 5 percent HCMV specific CD4+ T cells can be identified (Pitcher et al., 1999; Waldrop et al., 1997).

Relatively few authors have been able to characterise the memory phenotype of HCMV specific CD4+ T cells although Sester et al reported in a subset of 11 percent of normal individuals, where the HCMV response constitutes as much as 10-40 percent of the total response, the phenotype of these cells constitute an activated/memory phenotype, ($CD45RO^{Hi}$, CD27-, CD62L- and CCR7- expression) (Sester et al., 2002). Using an alternative technique, clonotypic oligonucleotide probing, Weekes has shown that pp65 peptide specific CD4+ T cells are both $CD45RA^{Hi}$ $CD45RO^{Lo}$ and $CD45RA^{Lo}CD45RO^{Hi}$ (Weekes et al., 2004), and Amyes has demonstrated that HCMV specific CD4+ T cells lack CD27 expression and heterogeneity with respect to CD28, while up to 20 percent of antigen experienced virus specific CD4+ cells can be both CD45RO+ and CD45 RA+ (Amyes et al., 2003).

The role for HCMV specific CD4+ T cell immunity as determined by interferon gamma production has been established in primary HCMV infection and also provides protection against HCMV disease in renal transplantation (Gamadia et al., 2003). Delayed recovery of HCMV specific CD4 function has been associated with symptomatic disease in renal transplant patients despite the presence of HCMV specific CD8+ cells (Sester et al., 2001; Gamadia et al., 2003), and may protect renal transplant recipients from high viral loads (Reusser et al., 1999). The role of HCMV specific CD4+ T cell functional assays in predicting patients at risk of HCMV infection following allogeneic stem cell transplantation remains to be evaluated.

4.2 Method

4.2.1 Patient Recruitment

Patients were recruited at the time of admission for allogeneic stem cell transplantation. Patient information sheets were provided, and written informed consent obtained prior to entry into the study. In the case of patients under the age of 18, informed consent was obtained from the parents. For patients Gilleck competent, consent was obtained from both the patient and the parents. A copy of the consent form and patient information sheet is included in Appendix A. The Royal Free Hospital ethics committee approved the study.

4.2.2 Samples

20 ml of peripheral blood was taken into a universal container containing preservative free heparin at two weekly intervals whilst the patient was an inpatient or at the time of an outpatient appointment. A full blood count sample and HCMV PCR sample were also obtained at the same time.

4.2.3 Viral Load Monitoring

HCMV PCR was monitored from whole blood twice weekly whilst the patient was an inpatient and then at each subsequent outpatient appointment. DNA was extracted using the method described in section 3.1.2, and TaqMan real time PCR for the HCMV viral load as per method described in section 3.1.3. Treatment of HCMV infection with either ganciclovir (5mg/kg BD), foscarnet (90mg/kg BD), combination ganciclovir (2.5mg OD)/foscarnet (90mg/kg OD) or valganciclovir (900mg BD) was initiated when the viral load was greater than 3000 copies/ml or

following two consecutive positive HCMV PCR results, and continued until two negative HCMV PCR results were obtained.

4.2.4 Conditioning Details and Infection Prophylaxis

Details of conditioning regimens and infection prophylaxis are detailed in section 2.2.2 and 2.2.3.

4.2.5 Clinical Data

Data on clinical outcome was obtained from the patient notes, including conditioning details, presence of GVHD, immunosuppressive and steroid use as well as details of treatment of HCMV infection.

4.2.6 Mononuclear Cell Isolation

In a laminar flow HEPA filtered hood, 20ml of heparinised whole blood was diluted 1:1 with Hank's balanced salt solution (HBSS, Gibco) and layered over Lymphoprep (Nycomed, Norway) at a ratio of 15ml of lymphoprep to 20 ml of diluted blood. This was centrifuged at 400G for 25 minutes with the brake off. The buffy layer was removed using a Pasteur pipette, placed in a clean 50ml Falcon tub and diluted to 50ml with HBSS. The suspension was then centrifuged at 400G for 10 minutes, and the supernatant discarded. The pellet was resuspended in 10ml of HBSS, mixed and 10µL removed for a cell count. The cell suspension was made up to 50ml and centrifuged at 200G for 10 minutes. The supernatant was discarded, the pellet resuspended and sufficient RPMI 1640 culture medium with Glutamax (Gibco) containing 100U/ml penicillin, 100µg/ml

streptomycin, and 10% heat inactivated Fetal Calf Serum (FCS) was added to make the suspension to 1-2 X 10^6 PBMCs per ml.

A cell count was performed by adding 10µL of trypan blue 0.4% (Sigma) to the 10µL cell aliquot of cell suspension in a 1.5 ml eppendorf tube and vortexing for 2 seconds. Counting was performed using a Neubaeur counting chamber.

4.2.7 Antigen Stimulation

Antigen stimulation of the mononuclear cell suspension was performed under sterile conditions in a laminar flow hood. In the case of the HCMV AD169 whole viral lysate response, a 1ml aliquot of PBMCs suspended in RPMI at a concentration of 1-2 X 10^6/ml was added to a 5ml polypropylene tube (Kendall). 5µg of commercially available sucrose gradient purified HCMV viral lysate (ABI) prepared from AD169 infected human fetal foreskin fibroblasts (ABI) was used as the source of antigen stimulation. A control assay using lysate prepared from uninfected human fetal foreskin fibroblasts was also prepared. 2 µL (1µg) of a monoclonal antibody to CD28 (BD) (low azide) was added for antigen co stimulation. After one hour incubation at a 5˚ slant, 37˚C, 50% humidity and 5% CO_2 in an incubator to allow macrophage processing of the antigen protein, 2 µL of GolgiStop (BD), containing brefeldin, was added to prevent interferon gamma secretion by blocking intracellular protein export. The phorbol ester, PMA (phorbol 12-myristate-13-acetate, Sigma) at a concentration of 50ng/ml (Sigma) with calcium ionophore [250ng/ml] (Sigma) was used as the positive control.

IE1 and pp65 peptide pools were stored at -22º C in 1 µg aliquots dissolved in 2.5 µL of DMSO. For HCMV IE1 and pp65 peptide pool responses, 0.5mL of PBMCs suspended in RPMI was added to each polypropylene tube and 1µg of the respective peptide pool was added. A negative control without peptide was included. The positive control used for the peptide antigen stimulation was 0.5 µg of SEB (Staphylococcal enterotoxin B, Sigma).1 µL of GolgiStop was added to each tube and the cells were incubated at a 5˚ slant, 37˚C, 50% humidity and 5% CO_2 for between 12 and 14 hours.

4.2.8 Intracellular Staining

Following stimulation, the cells were washed by adding 2ml of HBSS, centrifuging at 200G for 5 minutes, and the supernatant was discarded. After resuspending the cells, 5 µL of the appropriate conjugated monoclonal antibodies (BD) for surface markers: CD3 PerCP (peridin chlorophyll protein), CD69 PE (phycoerythrin) and CD4 or CD3 FITC (fluorescein isothiocyanin) for antigen responses, CD62L FITC, CD25 PE, CD45 RA FITC, CD45 RO FITC, and CD27 PE, for memory phenotype. HBSS 25 µL was added to each tube, vortexed for 2 seconds, and incubated in the dark for 10 minutes at room temperature. 100µL of intrastain reagent A (containing paraformaldehyde) (Dako) was added in order to fix the sample. The suspension was again vortexed, incubated for a further 15 minutes in the dark at room temperature, 2ml of HBSS was added, centrifuged for 5 minutes at 200G and supernatant was discarded, and the cell pellet was resuspended. Intracellular staining for interferon gamma was then performed by adding interferon gamma

monoclonal antibody conjugated to APC (Allophycocyanin, BD) 1 µL, and 100µL of reagent B (Dako) for cell permeabilisation. Following a further 15 minute incubation at room temperature, the suspension was again washed in 2ml HBSS, centrifuged for 5 minutes at 200G, the supernatant was discarded, and the cell pellet resuspended in 500µL of FACS flow medium (BD).The sample was then acquired in a four colour (six parameter) FACSCalibur (BD Bioscience) flow cytometer.

4.2.9 FACS Analysis

FACS analysis was performed using CellQuest software (BD Bioscience). For analysis of intracellular interferon gamma production, a lymphocyte gate was drawn around the live lymphocyte population on the FCS (Forward Scatter, determined by cell volume) versus SSC (Side scatter, determined by internal cell structure) plot. This gate was used to draw a density plot of CD3 versus CD4 or CD8, and the CD3+ CD4+ or CD3+ CD8+ populations were defined, taking care to include the $CD3^{low}$ population to account for downregulation of CD3 by antigen stimulated cells. The proportion of CD3+ CD4+ or CD3+ CD8+ cells staining for interferon gamma was calculated from the CD69 versus Interferon gamma plot, using CD69 upregulation to confirm interferon gamma positive cells had undergone activation. In order to control for background interferon gamma production, the percentage of interferon gamma positive cells in the negative control for peptide stimulated cells and the uninfected lysate control for the HCMV lysate stimulated cells was subtracted from the result. A positive control using either PMA or SEB was included. A target of acquiring 50,000 CD4 + cells was made in order

to acquire sufficient number of cells, but due to low CD4+ or CD8+ specific immune recovery following allogeneic stem cell transplant, this was not always possible in all patients.

The absolute CD4+ and CD8+ counts were calculated from the percentage of cells expressing CD3+ and CD4+ or CD3+ and CD8+ antigens on the live lymphocyte gate as a proportion of the total lymphocyte population and multiplying this by the absolute lymphocyte count.

4.2.10 Statistical Analysis

Statistical analysis was performed using SPSS Version12.1 and GraphPad Prism.

4.3 Results

4.3.1 Patients and Clinical Data

Twenty patients were recruited for monitoring of HCMV specific immune function following allogeneic stem cell transplantation. The median age was 45.5 years (range 9 to 60 years).The patient characteristics and conditioning details are summarised in Table 4-1.

Twenty healthy HCMV seropositive volunteers (lab workers) were used to establish a normal range of HCMV specific immune responses. Five HCMV seronegative volunteers were used as negative controls.

Table 4-1 Characteristics and conditioning details of patients recruited for HCMV specific immune function monitoring following allogeneic stem cell transplant.

Patient	*Disease*	*Age, years*	*Sex*	*HCMV serology (R/D)*	*Stem cell source*	*Cond-itioning*	*Campath -1H in vivo/in the bag*	*GvHD prophy-laxis*	*Follow -up days*
1P	ALL	19	M	pos/neg	MUD PBSC	Flu, Cyclo & TBI	No/Yes	None	129
8P	EP	60	M	pos/neg	MUD PBSC	Flu & Melph	Yes/No	None	299
2P	MM	49	F	pos/neg	Sib Mini-allo PBSC	Flu & Melph	Yes/No	None	336
15P	AML	35	F	pos/neg	Sib BM	Cyclo & TBI	No/No	MTX & CsA	362
3P	AML	47	M	pos/neg	Sib PBSC	Cyclo & TBI	No/Yes	None	208
17P	NHL	56	M	pos/neg	MUD Mini-allo PBSC	Flu & Melph	Yes/No	CsA	389
7P	CML	57	F	pos/pos	MUD PBSC	Flu & Melph	Yes/No	CsA	473
19P	ALL	27	M	pos/pos	Sib BM	TBI	No/No	CsA	142
4P	AML	32	M	pos/pos	Sib Mini-allo PBSC	Flu & Melph	Yes/No	None	325
5P	AML	9	M	pos/pos	Sib PBSC	Cyclo & TBI	No/No	MTX & CsA	256
12P	HL	19	F	pos/pos	Sib Mini-allo PBSC	Flu & Melph	Yes/No	None	188
11P	CLL	53	M	pos/pos	Sib Mini-allo PBSC	Flu & Melph	Yes/No	None	114
13P	AML	58	F	pos/pos	MUD PBSC	Flu & Melph	Yes/No	None	417
6P	ALL	17	M	pos/pos	Sib PBSC	Cyclo & TBI	No/No	MTX & CsA	166
18P	AML	44	M	pos/pos	Sib PBSC	Cyclo & TBI	No/No	MTX & CsA	444
16P	NHL	53	M	neg/pos	Sib Mini-allo PBSC	Flu & Melph	Yes/No	CsA	188
22P	FL	33	M	pos/neg	Sib Mini-allo PBSC	Flu and Melph	Yes/No	CsA	353
21P	AML	35	F	neg/pos	Sib Mini-allo PBSC	Flu & Melph	Yes/No	CsA	160
20P	AML	55	F	pos/pos	MUD Mini-allo PBSC	Flu & Melph	Yes/No	CsA	60
23P	CML	54	M	neg/pos	MUD PBSC	Cyclo & TBI	No/No	CsA	78

AML acute myeloid leukaemia; ALL acute lymphoblastic leukaemia; NHL non-Hodgkin's lymphoma; CML chronic myeloid leukaemia; HL Hodgkin's lymphoma; CLL chronic lymphocytic leukaemia; MM multiple myeloma; EP erythropoietic porphyria; FL fas ligand deficiency; MUD matched unrelated donor; PBSC peripheral blood stem cell: Sib sibling: MTX methotrexate; CsA ciclosporin; Cyclo cyclophosphamide; TBI total body irradiation; Flu fludarabine; Melph melphalan.

4.3.2 Results of FACS Analysis on Normal Controls

A typical FACS plot of the interferon gamma response to HCMV antigen stimulation is show in Figure 4-1.

In order to calculate the optimal quantity of HCMV AD169 viral lysate, a dose response curve of the CD4+ T cell interferon gamma response of 6 normal controls was generated, with the concentration of HCMV viral lysate ranging from 0.1ug/ml to 10ug/ml. The optimal concentration of the AD169 HCMV viral lysate was found to be 5ug/ml, with a reduction in the response in three individuals when the lysate was used at a higher dose, Figure 4-2.

The intra-assay variability was calculated by performing five replicates in two normal control subjects and was found to be 5%. It was not possible to calculate an inter-assay variability since deterioration in response with storage occurs. The importance of processing and setting up samples for stimulation within 12 hours was confirmed by the demonstration that the response to HCMV lysate fell by 50% in two patients after a 24 hour delay prior to antigen stimulation.

HCMV lysate or peptide pools did not elicit a significant response in five HCMV seronegative healthy controls, with the maximum interferon gamma response being 0.02% of CD4+ cells, which is the lower limit of detection for this assay.

The CD4+ T cell Interferon gamma responses to HCMV viral lysate was obtained in 20 healthy control subjects, and the CD4+ and CD8+ T cell interferon gamma responses to pp65 and IE1 peptide pools were obtained in 17 healthy HCMV seropositive control subjects and are

summarised in Table 4.2. The median CD4+ T cell response to the HCMV viral lysate was 3.88 interferon gamma secreting cells/μL, while the response to the pp65 peptide pool was 1.98 interferon gamma secreting cells/μL and to the IE1 peptide pool was 0.26 interferon gamma secreting cells/μL. The median CD8+ CTL interferon gamma response to pp65 peptide pool was 1.57 interferon gamma secreting cells/μL and the IE1 peptide pool was 0.5 interferon gamma secreting cells/μL.

Table 4-2 Interferon γ response of normal controls to stimulation with HCMV lysate, pp65 peptide pool, and IE1 peptide pool.

	Median Interferon Gamma Response	*Range*
Absolute Lymphocyte Count ($X10^9$)	1.85	1.15-2.84
CD4 Response		
Absolute CD4+ Count ($X10^9$)	0.84	0.41-1.53
% HCMV Lysate Response, N=20	*0.49*	*0.03-2.74*
HCMV Lysate Response cells/μL	3.88	0.11-30.84
% pp65 Peptide Pool Response, N=17	*0.22*	*0-0.51*
pp65 Peptide Pool Response cells/μL	1.98	0-5.08
% IE1 Peptide Pool Response, N=17	*0.03*	*0-0.11*
IE1 Peptide Pool Response cells/μL	0.26	0-0.97
CD8 Response		
Absolute CD8+ Count ($X10^9$)	0.47	0.26-1.08
% pp65 Peptide Pool Response, N=17	*0.34*	*0.05-3.98*
pp65 Peptide Pool Response cells/μL	1.57	0-10.36
% IE1 Peptide Pool Response, N=17	*0.16*	*0-2.95*
IE1 Peptide Pool Response cells/μL	0.50	0-10.94

Figure 4-1 Representative FACS plots of intracellular staining for interferon γ of CD4+ T cells following antigen stimulation.

R1 represents the live lymphocyte gate. R2 is gated around CD3+ CD4+ cells. CD3+CD4+ cells were defined by combining the R1 and R2 gates. The proportion of CD3+CD4+ cells producing interferon γ in response to specific antigen stimulation was determined on a quadrant plot of CD69 PE versus interferon gamma APC, with positive cells showing upregulation of CD69. Percentage of responding cells were defined as the proportion of cells in the right upper quadrant. Backgating on the interferon γ positive cells (coloured blue), it can be seen on the CD3 PerCP versus CD4 FITC plot that the surface expression of both are down regulated in antigen responding cells. Plots are shown for uninfected (control) lysate, HCMV infected lysate, no peptide control, pp65 peptide pool, IE1 peptide pool and SEB positive control.

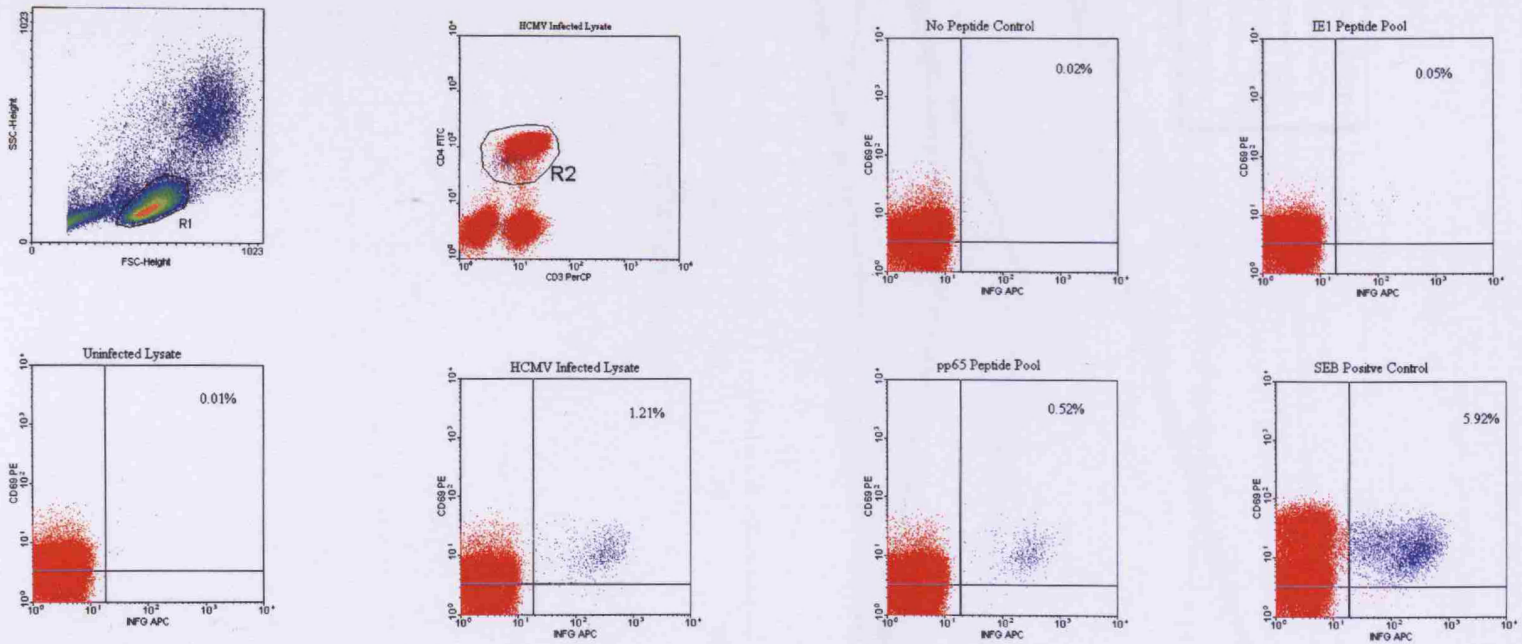
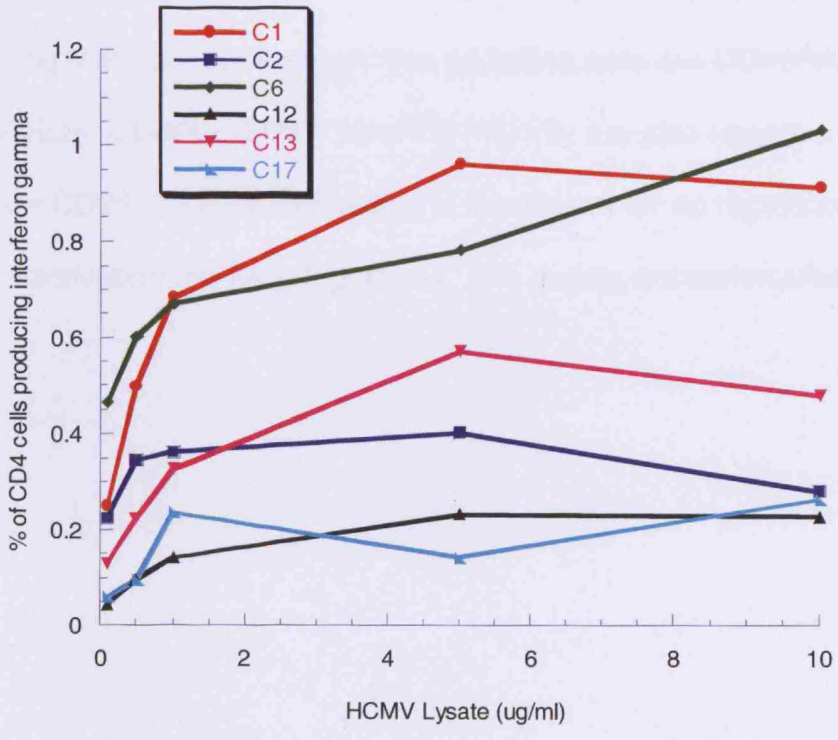


Figure 4-2 HCMV viral lysate dose response curve.

The percentage of CD4+ T cells producing interferon γ in response to stimulation with HCMV viral lysate at the concentrations 0.1, 0.5, 1, 5 and 10 μg/ml is shown for the 6 normal controls, C1, C2, C6, C12, C13 and C17. The optimal concentration of HCMV viral lysate was determined to be 5 μg/ml. In three healthy controls, a reduction in response was seen when 10 μg/ml was used.



4.3.3 Immunophenotype of CD4+ T cells Responding to HCMV

The immunophenotype of CD4+ T cells responding to HCMV AD169 viral lysate was determined and a representative plot from a normal HCMV seropositive control is shown in Figure 4-3. The terminally differentiated mature phenotype was confirmed in 2 normal controls and 5 patients, showing that the interferon gamma secreting cells are CD45RA-, CD45RO+, CD62L-, CD27-, and the majority are also negative for the marker CD25 (12 hour stimulation is insufficient for up regulation of CD25 as an activation marker), Figure 4-4. The results are summarised in Table 4-3.

Figure 4-3 Representative FACS plot of the immunophenotype of CD4+ T cells responding to HCMV viral lysate.

The results of immunophenotyping of patient 2P following stimulation with HCMV viral lysate is shown. R1 represents the live lymphocyte population. R2 is gated around CD4+ lymphocytes, and CD4+ T cells are defined by combining R1 and R2. The second row shows the CD62L, CD25PE, CD45RA, and CD45RO versus intracellular interferon γ plots for CD4+ T cells with positive cells coloured blue. The third row shows the respective frequency histograms for interferon γ positive cells and the bottom row the frequency histograms for all CD4+ lymphocytes. The percentage positive (M1) and negative (M2) cells are indicated for each respective marker.

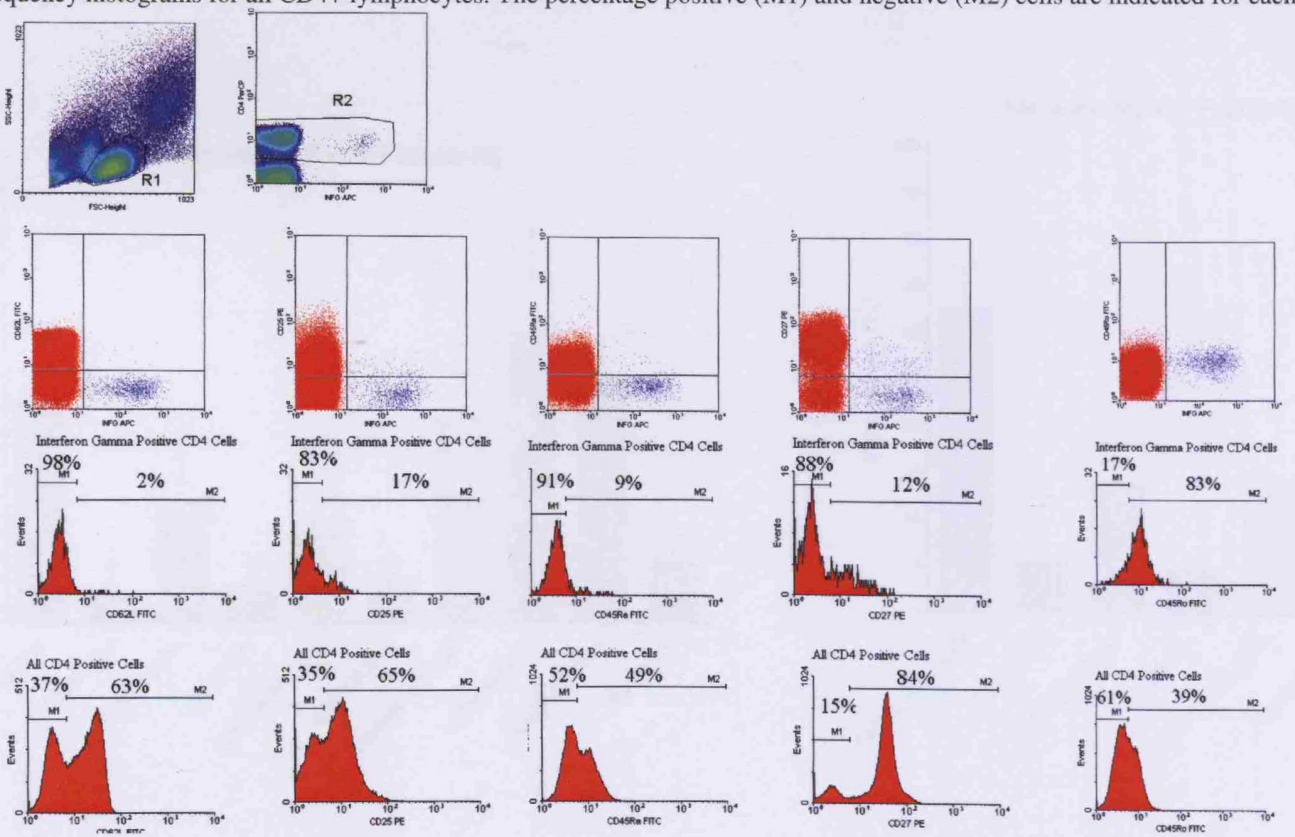
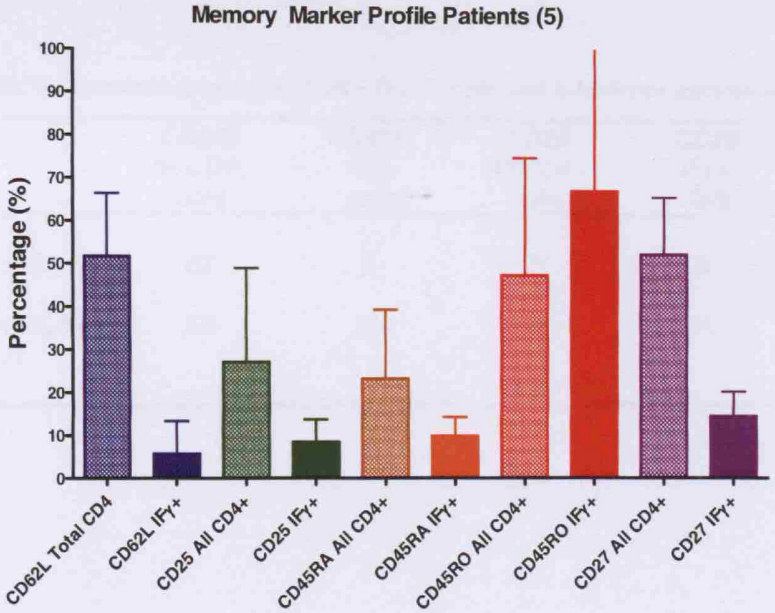


Figure 4-4 Comparison of the immunophenotype of the total CD4+ T cell population and CD4 T+ cells responding to HCMV viral lysate.

Bar graphs of the immunophenotype of all CD4+ T cells, and CD4+ T cells producing interferon γ in response to stimulation with HCMV viral lysate are shown. The bar graph on the left shows the mean results for the five patients 2P, 12P, 13P, 15P, and 18P, and the bar graph on the right shows the mean results for the two normal controls 1P and 2P. The hatched bars represent all CD4+ T cells and the solid bars represent CD4+ T cells producing interferon γ in response to stimulation with HCMV viral lysate. The mean percentage of cells positive for the markers CD62L, CD25, CD45RA, CD45R0 and CD27 is shown, with the error bars indicating 1 standard deviation.



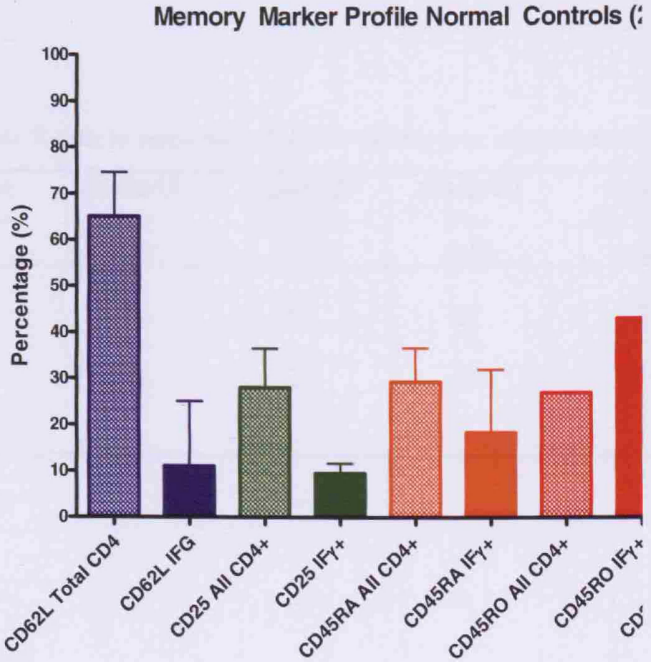


Table 4-3 Immunophenotype of all CD4+ T cells and interferon gamma secreting CD4+ T cells in response to HCMV viral lysate, of patients and controls.

	CD62L *All CD4+ cells*	***CD62L*** *IFγ+ cells*	***CD25*** *All CD4+ cells*	***CD25*** *IFγ+ Cells*	***CD45RA*** *All CD4+ cells*	***CD45RA*** *IFγ+ cells*	***CD45RO*** *All CD4+ cells*	***CD45RO*** *IFγ+ cells*	***CD27*** *All CD4+ cells*	***CD27*** *IFγ+ cells*
Patients, % (N=5)	52	6	27	8	23	10	47	67	52	14
Control, % (n=2)	65	11	28	10	29	18	27	43	81	37

4.3.4 Results of HCMV Specific Immune Function Monitoring in Allogeneic Stem Cell Transplant Recipients

All twenty patients were monitored for CD4+ and CD8+ T-cell HCMV specific immune response by interferon gamma secretion in response to stimulation with AD169 HCMV viral lysate (CD4+ only), and the pp65 and IE1 peptide pools (CD4+ and CD8+ responses). Patients were monitored for a median of 232 days post transplantation (range 60 to 444 days).The median number of samples per patient was 10.5 (range 3 to 17).

A representative plot of the recovery of the CD4+ T helper HCMV specific immune recovery (determined by interferon gamma secretion in response to HCMV AD169 viral lysate) and CD8+ T cells (determined by interferon gamma secretion in response to the IE1 and pp65 peptide pools), as well as real time PCR HCMV viral load monitoring is shown in Figure 4-5.

A scatter plot of the percentage of the total and $\log_{10}$(interferon gamma secreting cells/μL) CD4+ T helper cells responding to HCMV viral lysate, pp65 peptide pool, and IE1 peptide pool versus time post transplant, along with the results from normal controls is shown in Figure 4-6. By 60 days post transplant, the median percentage of the total CD4 T cell population responding to HCMV viral lysate exceeds that of normal controls, indicating that the HCMV specific response is a dominant component of early CD4+ T cell immune reconstitution post transplant. The absolute interferon gamma response to HCMV viral lysate steadily increases following transplant, but only approaches similar levels to normal controls by 1 year post transplant. Although many of the

allogeneic stem cell transplant recipients achieve an absolute CD4+ T cell pp65 peptide pool response similar to normal controls by day 90, recovery of IE1 peptide pool CD4 responses is slower, not reaching similar levels to normal controls until day 360 post transplant.

The Kaplan Meier estimate of the cumulative incidence of HCMV specific CD4+ T cell response to HCMV viral lysate greater than one interferon gamma secreting cell/μL is 57% of patients with a median time of 178 days.

A scatter plot of the percentage of the total and $\log_{10}$(interferon gamma secreting cells/μL) CD8+ T cells responding to pp65 and IE1 peptide pools versus time post transplant, and the results from normal controls is shown in Figure 4-7. The CD8 pp65 peptide pool response is recovered rapidly following transplantation, with some patients having a significant level of response as early as day 30 post transplant. The CD8+ T cell response to pp65 dominates the early CD8 T cell immune response, with a median of 4% of the total CD8+ T-cells responding to pp65 at Day 30 post transplant, ranging from 0% to as high as 64.1% in one patient. By day 90 post transplant, the median absolute CD8+ T cell response to pp65 exceeds that of the normal control population, and remains greater than the normal controls at 1 year post transplant. Recovery of the CD8+ T cell response to the IE1 peptide pool is slower and although the median percentage of the total CD8+ T cell population exceeds that of the normal control population by day 90, the absolute CD8 IE1 response does not reach similar levels to normal controls until day 270 post transplant. The Kaplan Meier estimate of the cumulative incidence of the CD8+ T cell

response to pp65 peptide pool greater than 1 interferon gamma secreting cell/μL is 84.6% with a median recovery time of 94 days.

A bar graph of the median and interquartile range of the absolute interferon gamma response to HCMV viral lysate (CD4+ T cells) as well as the pp65 and IE1 peptide pools (CD4+ and CD8+ cells T cells) versus time post transplant is shown in Figure 4-8. This graph confirms that pp65 dominates the early HCMV response for both CD4+ and CD8+ T cells.

Figure 4-5 Representative plot of the HCMV specific immune response and viral load monitoring following transplant.

The FACs plots show the percent of CD4+ T cells producing interferon γ (upper right quadrant) in response to stimulation with HCMV viral lysate. The red line on the line graph shows HCMV viral load monitoring (log_{10} genome copies/ml) versus time post transplant. The blue (CD4+ T cells), green (CD8+ T cells) and black (CD8+ T cells) lines show the number of interferon γ producing cells/μL following stimulation with HCMV viral lysate , pp65 and IE1 peptide pools respectively.

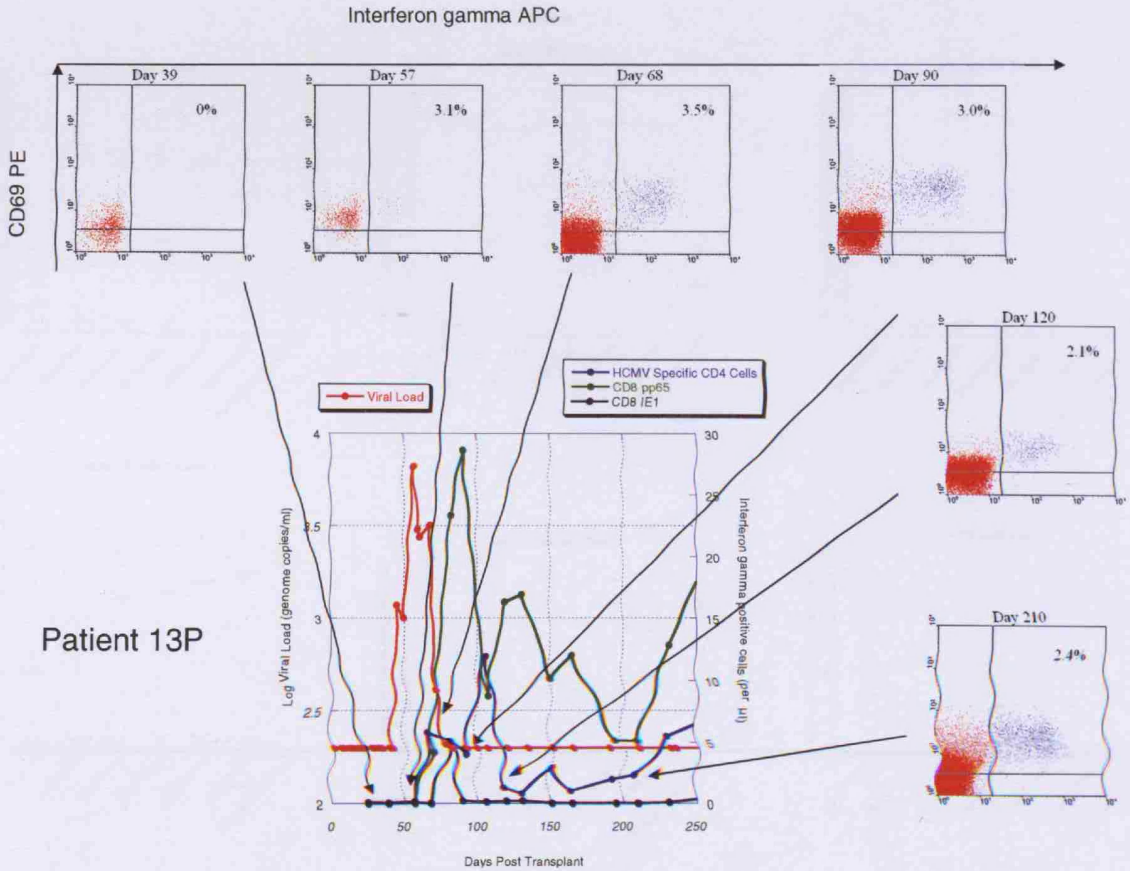


Figure 4-6 Scatter plots of the CD4+ T cell response to HCMV versus time post transplant.

The results of the CD4+ T cell interferon γ response to stimulation with HCMV viral lysate, pp65 and IE1 peptide pools in 20 allogeneic transplant recipients at risk of HCMV infection are plotted. The top row of scatter plots shows the response as a percentage of the total CD4+ lymphocyte population, and the bottom row of scatter plots shows the absolute interferon gamma response expressed as $\log_{10}$(interferon γ producing cells/mL).

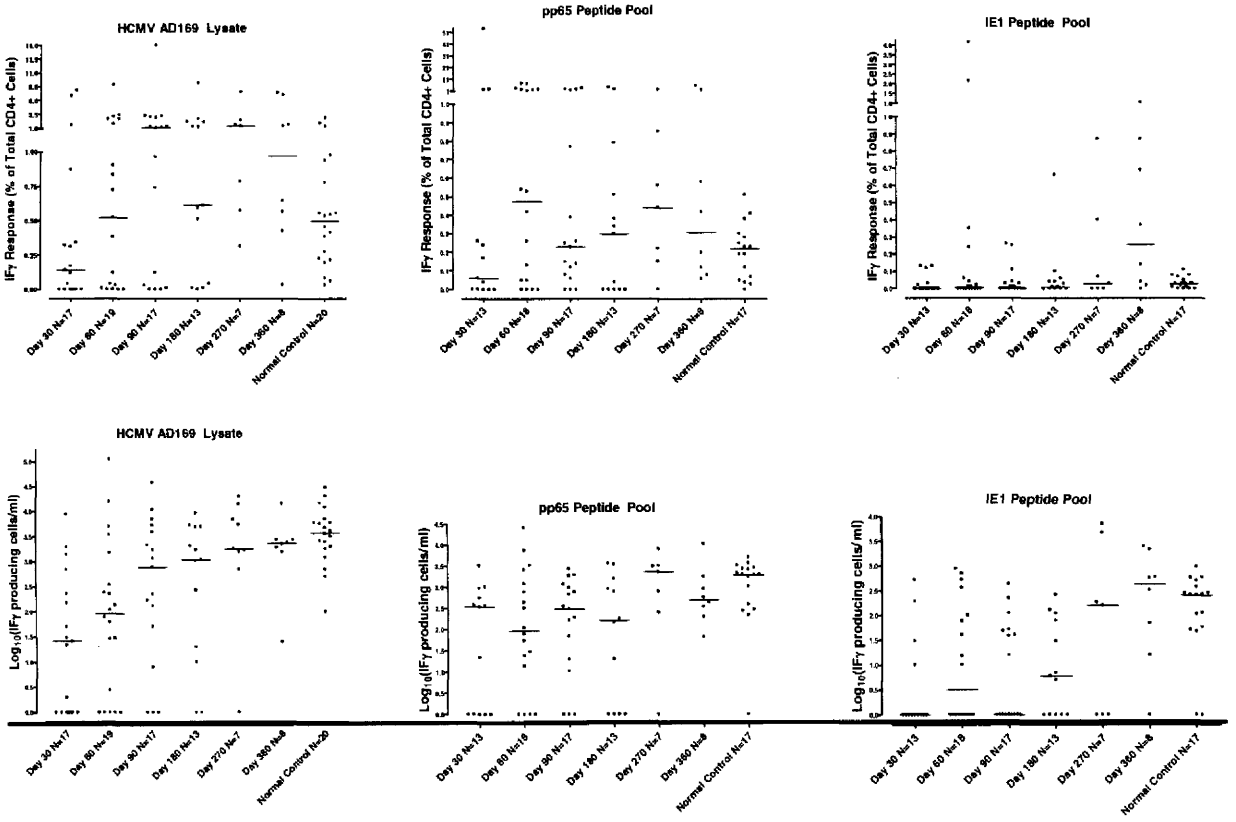


Figure 4-7 Scatter plots of CD8+ T cell response to HCMV versus time post transplant.

The results of the CD8+ T cell interferon γ response to stimulation with HCMV viral lysate, pp65 and IE1 peptide pools in 20 allogeneic transplant recipients at risk of HCMV infection are plotted. The top row of scatter plots shows the response as a percentage of the total CD4+ lymphocyte population, and the bottom row of scatter plots shows the absolute interferon gamma response expressed as $\log_{10}$(interferon γ producing cells/mL).

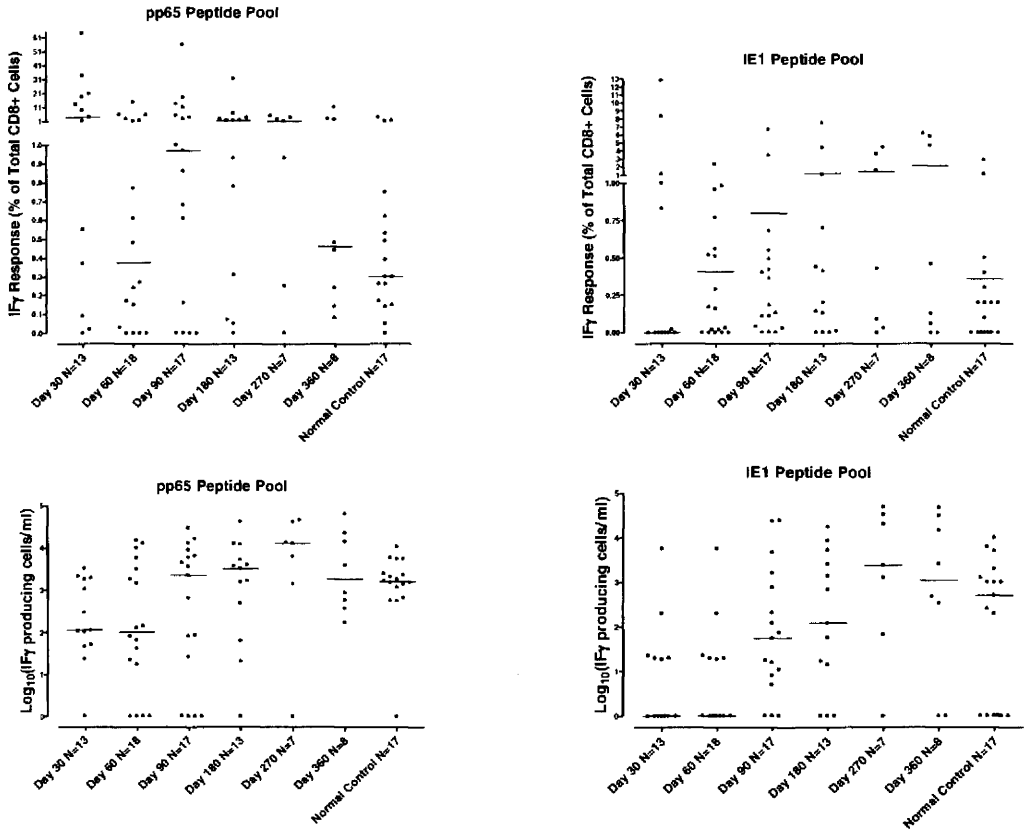
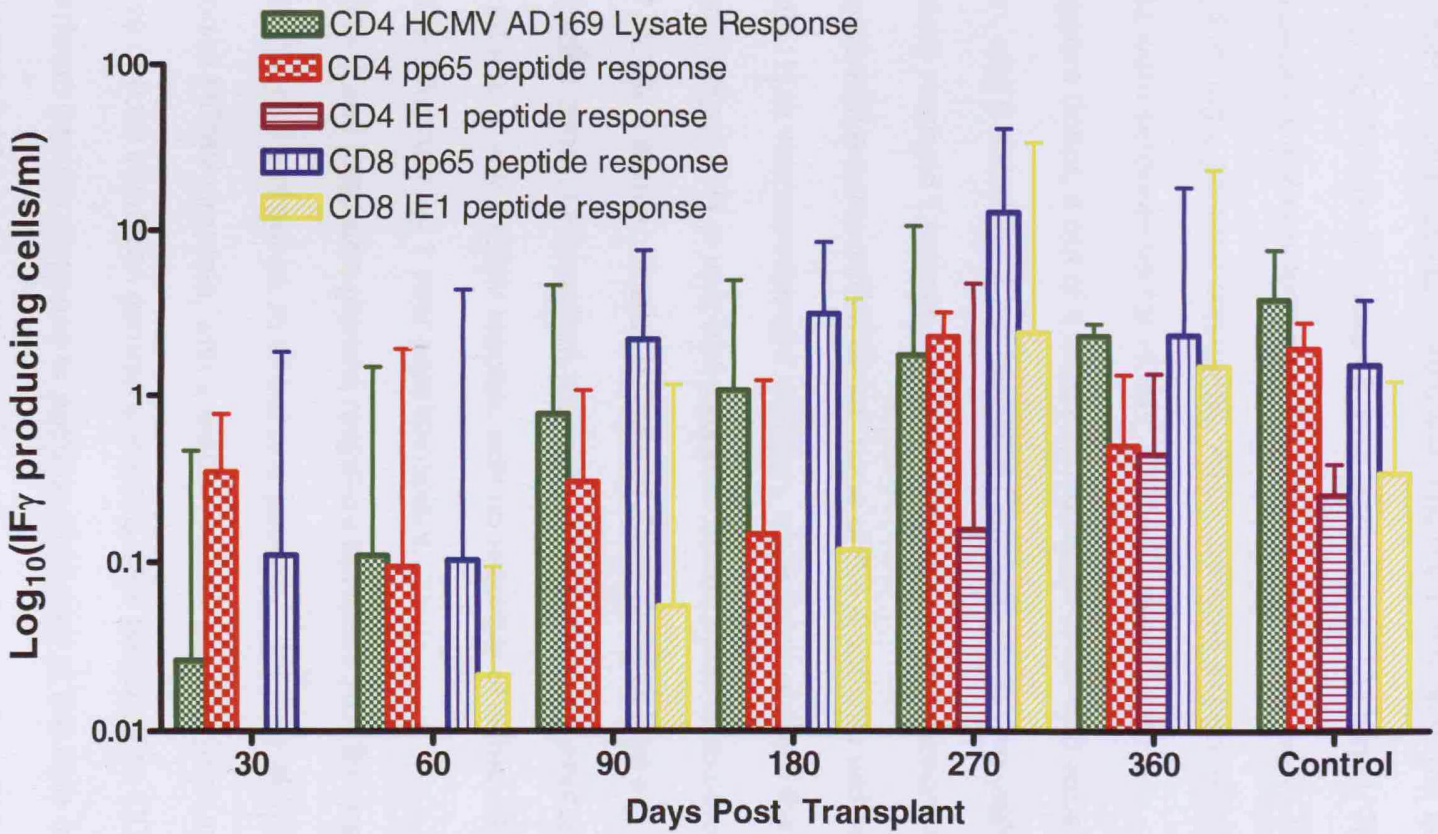


Figure 4-8 The CD4+ and CD8+ T cell interferon gamma response to HCMV post transplant.

The median and interquartile range of the CD4+ and CD8+ T cell interferon γ response to HCMV specific antigen stimulation for 20 allogeneic transplant patients is shown at 30, 60, 90, 180, 270 and 360 days post transplant. The results of 20 normal controls are plotted on the right hand side for comparison. Results are expressed as $\log_{10}$ (interferon γ producing cells/mL). The error bars indicate the interquartile range.



4.3.5 Correlation of Immune Function with HCMV Infection

Eleven of the twenty stem cell transplant recipients experienced 19 separate episodes of HCMV infection (as defined by two consecutive HCMV PCR positive results), Table 4-4. The median time to the first episode of HCMV infection was 46 days (range -1 to 134 days). The median peak HCMV viral load was 3.42 $\log_{10}$ genome copies/ml (range 2.38 to 4.95 $\log_{10}$ genome copies/ml). All the patients experiencing viraemia were seropositive for HCMV. Of the 7 patients with a seronegative donor, 4 out of 4 receiving Campath-1H in vivo experienced infection, and 2 out of the 3 not receiving Campath-1H in vivo (although one patient received Campath-1H ex vivo) experienced infection. Of the 10 patients with a seropositive donor, none of the 4 patients without Campath-1H in vivo experienced infection, while 5 out of the 6 that received Campath-1H in vivo had infection. Seven patients experienced a second HCMV infection, and all but one of these patients had a seronegative donor. Only patient (patient 17P) developed symptomatic HCMV infection, with HCMV retinitis, with no recovery of either CD4+ or CD8+ T cell function by 1 year post transplant.

The CD4+ T cell interferon gamma response to HCMV AD169 viral lysate was less the one cell per μL in all but one patient at the time of initial detection of HCMV viraemia, with a median HCMV specific immune response of 0.02 interferon gamma secreting cells per μL. The CD8+ T cell interferon gamma response to pp65 peptide pool at the time of HCMV viraemia was 0.13 interferon gamma secreting cells per μL, with a CD8+

T Cell response of greater than 1 per μL occurring in 5 episodes of viraemia. Kaplan Meier estimates of the time to recovery of a CD4+ T cell HCMV viral lysate response greater than one cell per μL according to the presence or absence of HCMV infection shows that although the overall recovery of HCMV specific CD4+ T cell immune responses is similar (55±34.2% for patients experiencing infection versus 58±33.9% for patients not experiencing infection, p=0.72 Log Rank Score), recovery is slower in patients who experience infection, Figure 4-9. The median time to HCMV specific CD4+ T cell recovery was 94 days in the group without infection, and 178 days in the group with infection, with none of the patients with infection experiencing a recovery of HCMV specific CD4+ T cell function until day 68. Although a greater proportion of patients in the HCMV infection group recovered an HCMV specific CD8+ T cell immune response to pp65 of greater than one cell per μL, the difference was not significant (87.9±21.6% for the patients experiencing infection versus 58.3±33.9% for the patients not experiencing infection, p=0.86 Log Rank Score), Figure 4-10. The median time to HCMV specific CD8 T cell recovery was 94 days in the group without infection and 101 days in the group with infection.

The median and interquartile range of the immune responses to HCMV viral lysate (CD4+ only), pp65 peptide pool and IE1 peptide pool at days 30, 60, 90, 180, 270 and 360 post transplant according to the presence or absence of HCMV infection are shown for CD4+ T cells in Figure 4-11 and Figure 4-12, and for CD8+ T cells in Figure 4-13. Patients experiencing HCMV infection had a lower HCMV specific CD4+ T cell

lysate response prior to day 180 post transplant, with a similar but less marked trend for pp65 and IE1. In contrast, there was no difference in the CD8+ T cell pp65 and IE1 responses between patients experiencing HCMV infection compared to patients not experiencing infection.

Table 4-4 Viral kinetics and HCMV specific immune response at time of infection of the allogeneic transplant patients experiencing HCMV infection.

Patient	*HCMV Serology (R/D)*	*Campath-1H In vivo*	*Time to Infection (days)*	*Log_{10} Peak Viral Load (genome copies/ml)*	*Log_{10} Initial Viral Load (genome copies/ml)*	*Kmax ($days^{-1}$)*	*CD4 Lysate Response (IFγ/μl)*	*CD8 pp65 response (IFγ/μl)*	*Recurrent Infection*
1P	pos/neg	no*	28	3.19	2.84	0.14	0.03	0.29	yes
2P	pos/neg	yes	50	2.94	2.50	0.23	#	#	yes
3P	pos/neg	no	84	2.71	2.71	0.16	0	0.13	yes
7P	pos/pos	yes	134	3.42	2.80	0.24	3.6	3.57	no
8P	pos/neg	yes	110	3.47	2.73	0.57	0	0.09	yes
11P	pos/pos	yes	20	3.87	2.61	0.21	0.47	2.2	yes
12P	pos/pos	yes	20	4.95	2.65	0.52	0	0.02	no
13P	pos/pos	yes	46	3.82	3.07	0.36	0	0	no
17P	pos/neg	yes	71	3.42	2.79	0.19	0	0	yes
20P	pos/pos	yes	29	3.85	2.66	0.42	#	#	no
22P	pos/neg	yes	-1	2.38	2.38	0.06	0	0	yes

* Patient received Campath Ex Vivo

Immune response at the time of infection not available

Figure 4-9 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV.

The Kaplan Meier estimate of the cumulative incidence of patients achieving a CD4+ T cell interferon γ response to HCMV viral lysate of greater than 1 cell/μL is shown. The cumulative incidence for the group experiencing HCMV infection (n=11, red line) is 55±34.2%, and for the group not experiencing infection (n=9, blue line) is 58.3±33.9%. The difference is not significant (p=0.4 Log Rank Score). Dotted lines indicate the 95% confidence intervals and censored events are indicated by the cross bars.

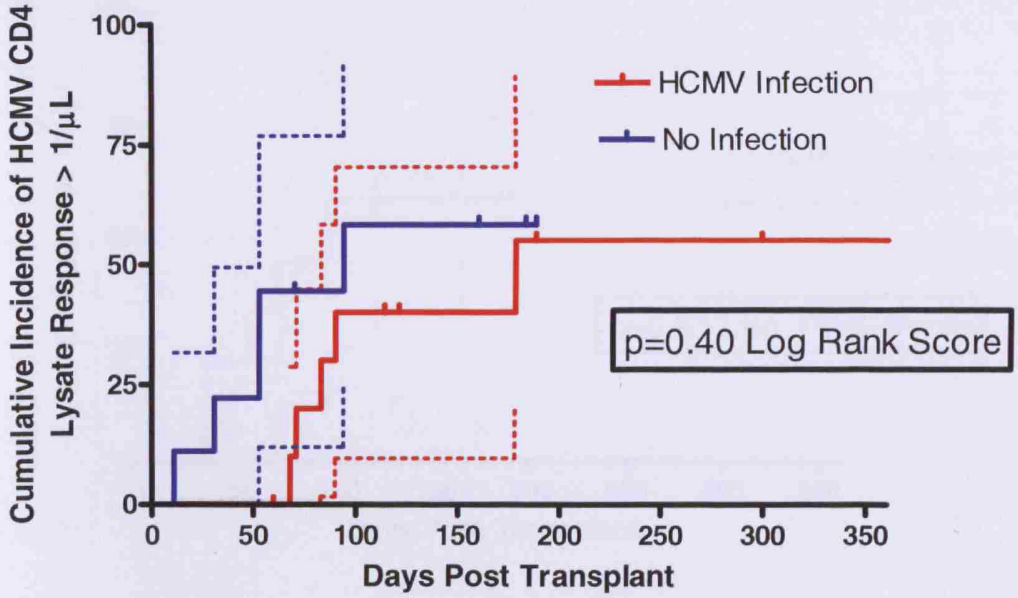


Figure 4-10 Kaplan Meier estimate of the cumulative incidence of CD8+ T cell immune recovery to HCMV.

The Kaplan Meier estimate of the cumulative incidence of patients achieving a CD8+ T cell interferon γ response to the pp65 peptide pool of greater than 1 cell/μL is shown. The cumulative incidence for the group experiencing HCMV infection (n=11, red line) is 87.9±21.6%, and for the group not experiencing infection (n=9, blue line) is 58.3±33.9%. The difference is not significant (p=0.85 Log Rank Score). Dotted lines indicate the 95% confidence intervals and censored events are indicated by the cross bars.

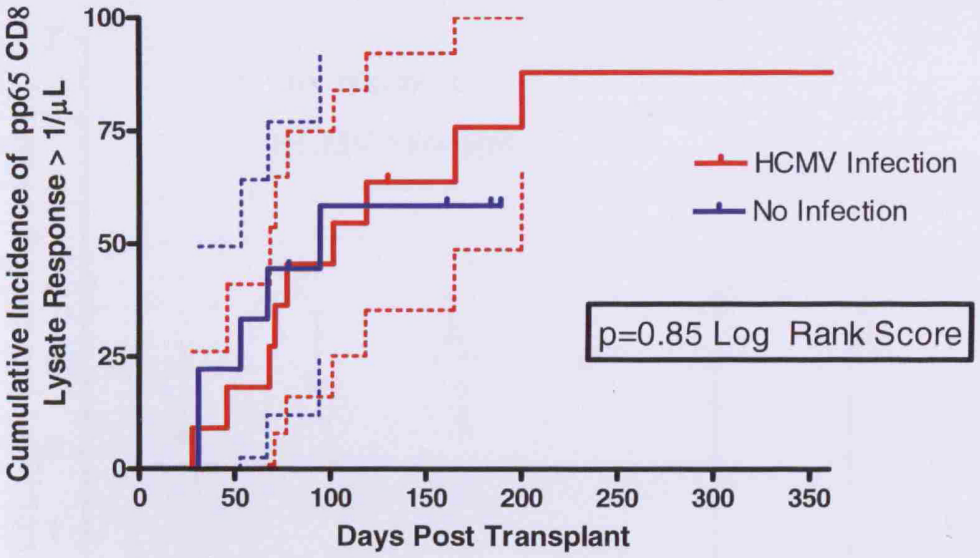


Figure 4-11 CD4+ T cell response to HCMV viral lysate according to the presence or absence of HCMV infection.

The $\log_{10}$ CD4+ T Cell interferon γ response versus time post transplant is plotted following stimulation with HCMV viral lysate. The results of patients experiencing infection (n=11) are plotted in red and patients not experiencing infection (n=9) are plotted in blue. The triangles/square boxes indicate the median values, and the error bars indicate the interquartile range.

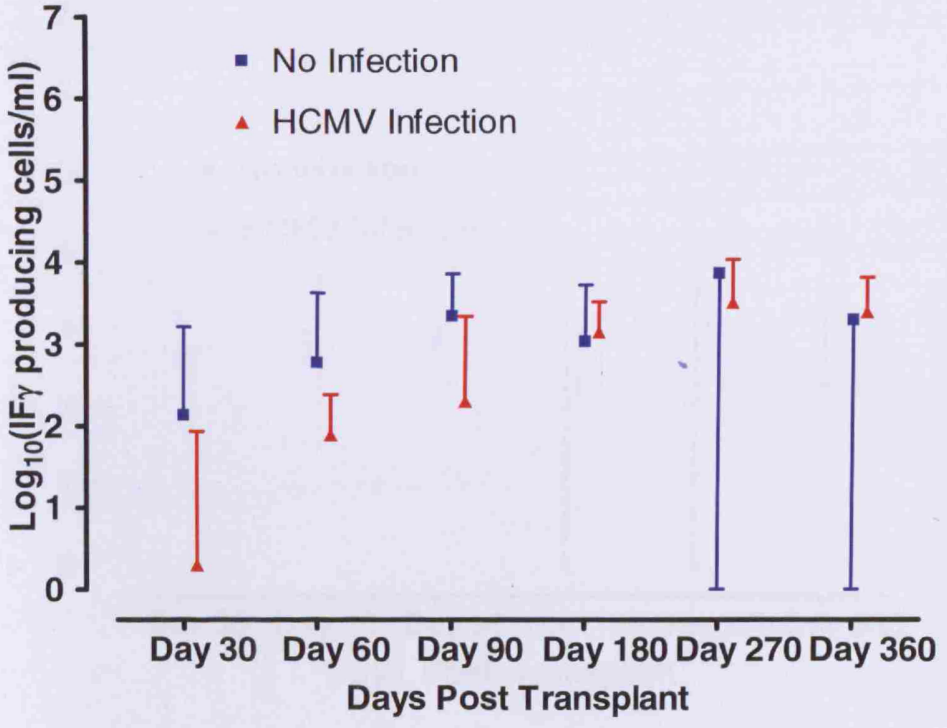
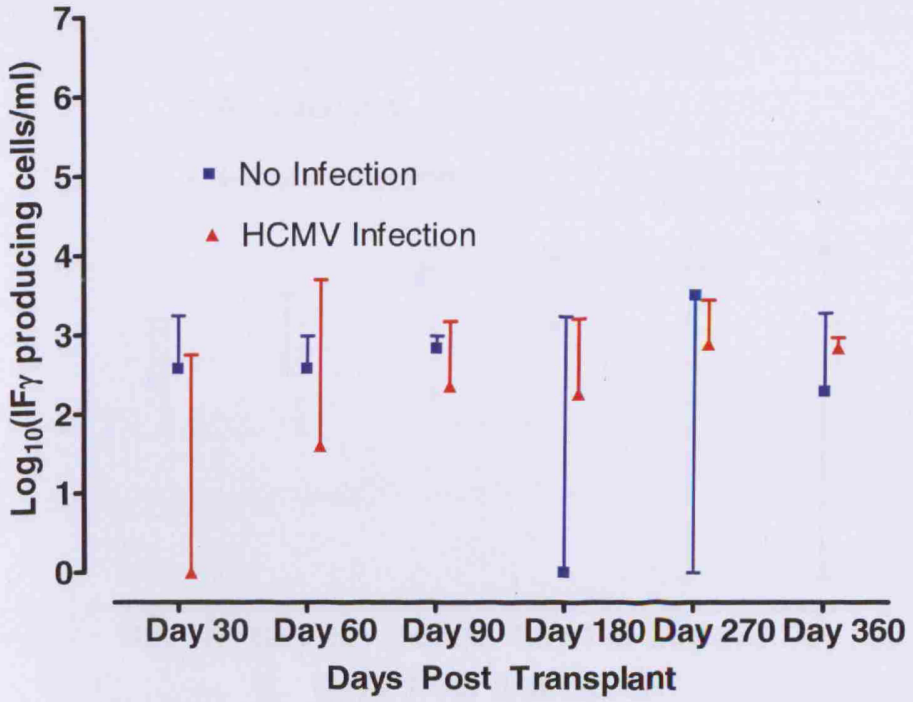


Figure 4-12 CD4+ T cell response to pp65 and IE1 peptide pools according to the presence or absence of HCMV infection.

The $\log_{10}$ CD4+ T Cell interferon γ response versus time post transplant is plotted following stimulation with the pp65 (top graph) and IE1 (bottom graph) peptide pools. The results of patients experiencing infection (n=11) are plotted in red and patients not experiencing infection (n=9) are plotted in blue. The triangles/square boxes indicate the median values, and the error bars indicate the interquartile range.



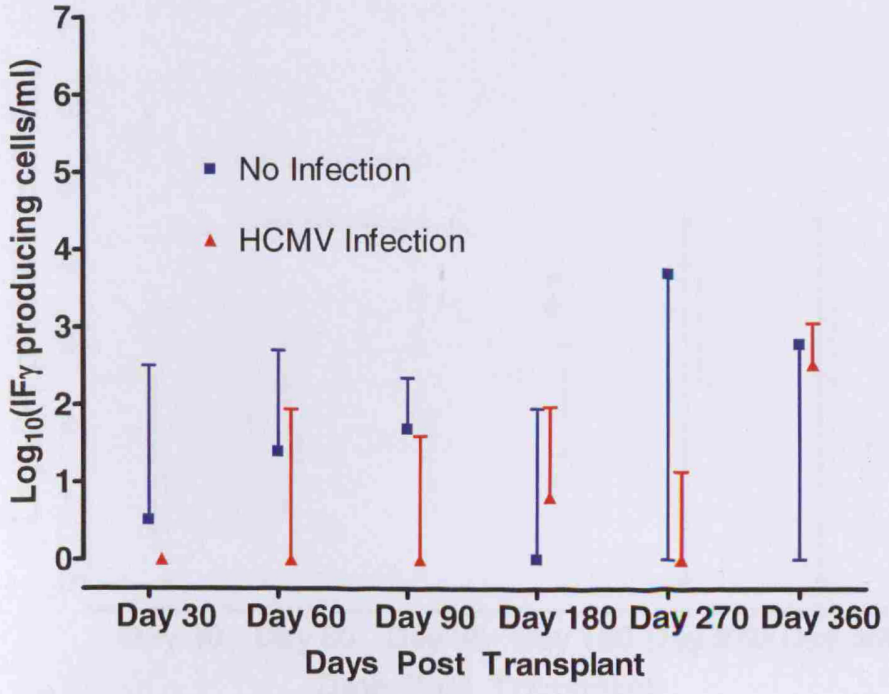
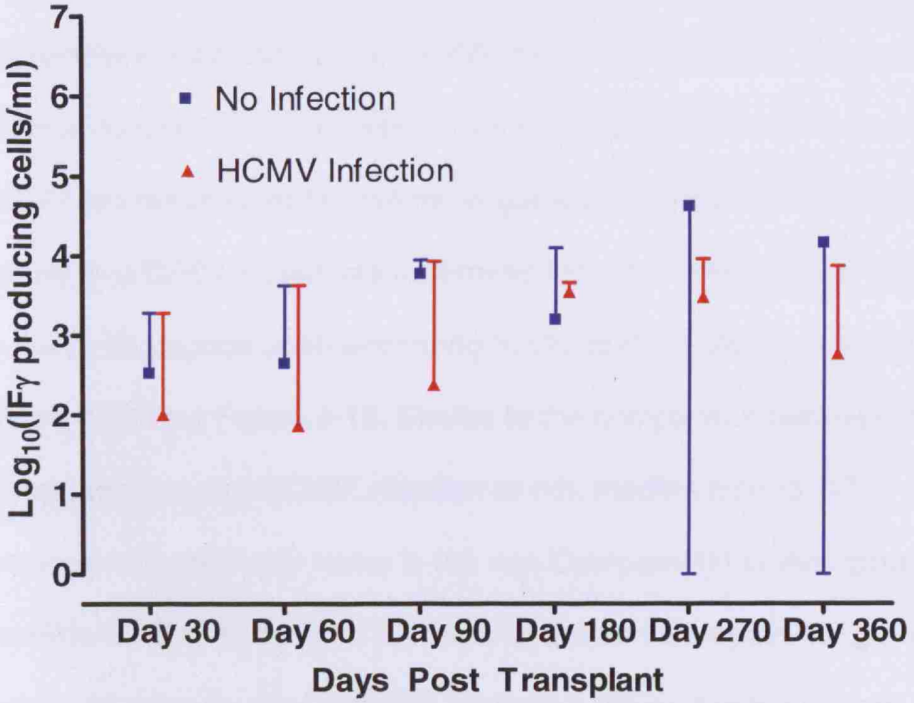
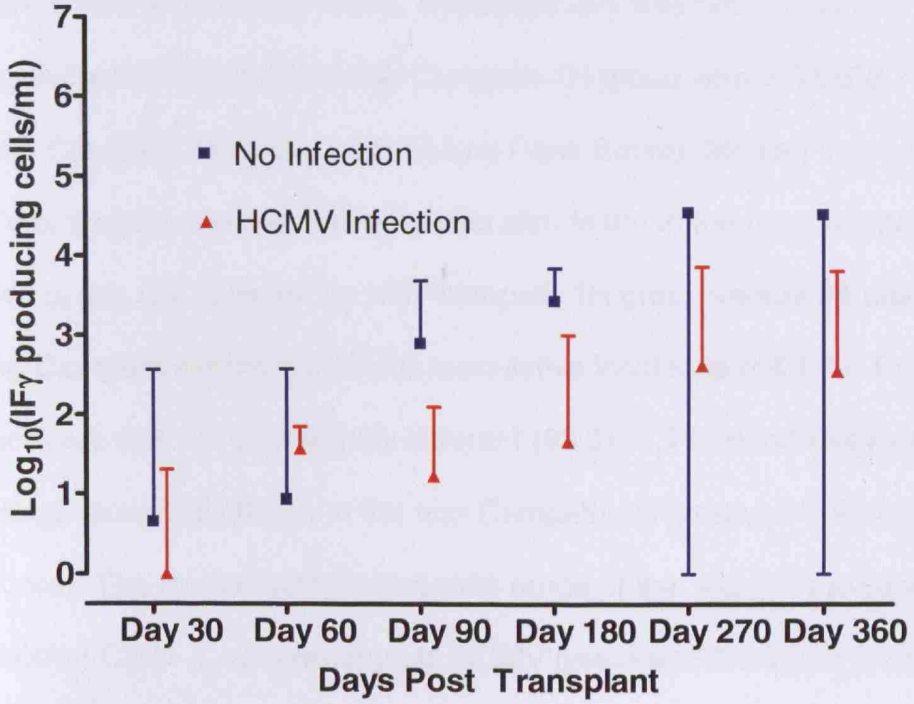


Figure 4-13 CD8+ T cell response to pp65 and IE1 peptide pools according to the presence or absence of HCMV infection.

The $\log_{10}$ CD8+ T cell interferon γ response versus time post transplant is plotted following stimulation with the pp65 (top graph) and IE1 (bottom graph) peptide pools. The results of patients experiencing infection (n=11) are plotted in red and patients not experiencing infection (n=9) are plotted in blue. The triangle/square boxes indicate the median values, and the error bars indicate the interquartile range.





4.3.6 Effect of Campath-1H Use on HCMV Immune Recovery

The use of Campath-1H in vivo was strongly associated with HCMV infection, with 9 out of the 10 patients (90%) who received Campath-1H in vivo experiencing infection, compared to only 2 out of 10 patients (20%) not receiving Campath-1H (p=0.006, Fisher's exact test). Kaplan Meier survival curves of the cumulative incidence of CD4+ T cell recovery to HCMV (as determined by interferon gamma response to HCMV viral lysate) and CD8+ T cells (as determined by interferon gamma response to the pp65 peptide pool) according to Campath In Vivo use are shown in Figure 4-14 and Figure 4-15. Similar to the comparison between the group experiencing HCMV infection or not, median time to CD4+ T cell recovery occurred was faster in the non Campath-1H in vivo group (median time to recovery of 53 days for the non Campath-1H group versus 94 days for the Campath-1H in vivo group) but the overall cumulative incidence of CD4+ T cell recovery was not statistically significant (54.2±29.5% in the Campath-1H group versus 75±38.7% in the Non Campath-1H group, p=0.33 Log Rank Score). Median time to CD8+ T cell specific immune recovery was also faster in the non Campath-1H in vivo group (67 days for the non Campath-1H group versus 94 days for the Campath-1H group) but the cumulative incidence of CD8+ T cell recovery was not significantly different (83.3±26.7% in the Campath-1H group versus 75±38.7% in the non Campath-1H group, p=0.83 Log Rank Score). The median and interquartile range of the $\log_{10}$(IFγ producing cells/μl) CD4+ T cell response to HCMV lysate and the CD8+ T cell

response to pp65 over time post transplant according to Campath-1H in vivo use is shown in Figure 4.15 and Figure 4.16, and the statistical analysis is shown in Table 4.5. Although the median CD4 response is lower at all time points post transplant in patients receiving Campath-1H in vivo, the difference is only significant at Day 30 (0.0 IFγ secreting cells/μL for the Campath-1H group and 0.7 IFγ secreting cells/μL for the non Campath-1H group, p=0.04 Mann Whitney U test). A similar analysis for CD8 responses to pp65 did not show any significant difference at any stage post transplant.

Table 4-5 Comparison of the median HCMV specific interferon gamma responses in allogeneic transplant recipients according to use of Campath-1H in vivo at different time points post transplant.

Days Post Transplant	*30*	*60*	*90*	*180*	*270*	*360*
Median CD4+ T cell Viral Lysate Response (cells/μL)						
Campath-1H	0	0.0	0.2	0.7	1.8	2.3
(Range)	(0, 0.2)	(0, 114)	(0, 38.8)	(0, 9.4)	(0.7, 20.3)	(0.0, 14.5)
No Campath-1H	0.70	0.9	3.8	5.0	10.7	2.3
(Range)	(0.0, 8.9)	(0, 16.0)	(0.1, 11.1)	(1.1, 5.0)	(7.1, 14.2)	(1.9, 2.8)
p value (Mann Whitney)	0.04	0.08	0.17	0.37	0.38	0.86
Median CD8+ T cell pp65 Response (cells/μL)						
Campath-1H	0.1	0.0	0.6	2.4	6.2	2.4
(Range)	(0, 3.1)	(0, 14.8)	(0, 28.6)	(0, 41.5)	(0, 40.6)	(0, 41.5)
No Campath-1H	1.0	0.8	4.7	3.7	29.4	8.9
(Range)	(0.1, 2.1)	(0, 12.6)	(0, 8.8)	(1.5, 11.9)	(13.2,45.7)	(3.8, 13.9)
p Value (Mann Whitney)	0.2	0.14	0.69	0.69	0.19	0.36

Figure 4-14 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV according to Campath-1H use.

Kaplan Meier estimate of the cumulative incidence of patients achieving a CD4+ T Cell interferon γ response greater than 1 cell/μL to HCMV viral lysate is plotted. The cumulative incidence for the Campath-1H group (n= 12, red line) is 54.2±29.5%, and for the non Campath-1H group (n=8, blue line) is 75±38.7%. The difference is not significant (p=0.33 Log Rank Score). Dotted lines indicate the 95% confidence intervals and censored events are indicated by the cross bars.

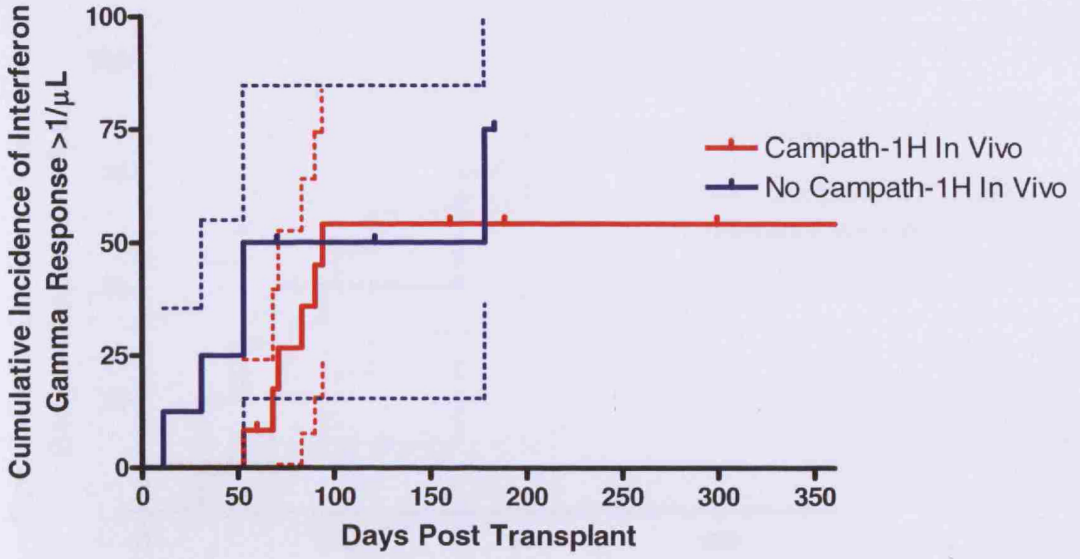


Figure 4-15 Kaplan Meier estimate of the cumulative incidence of CD8+ T cell immune recovery to HCMV.

The Kaplan Meier estimate of the cumulative incidence of patients achieving a CD8+ T Cell interferon γ response greater than 1 cell/μL in response to the pp65 peptide pool is plotted. The cumulative incidence for the Campath-1H group (n=12, red line) is 83.3±26.7%, and for the non Campath-1H group (n=8, blue line) is 75±38.7%. The difference is not significant (p=0.33 Log Rank Score). Dotted lines indicate the 95% confidence intervals and censored events are indicated by the cross bars.

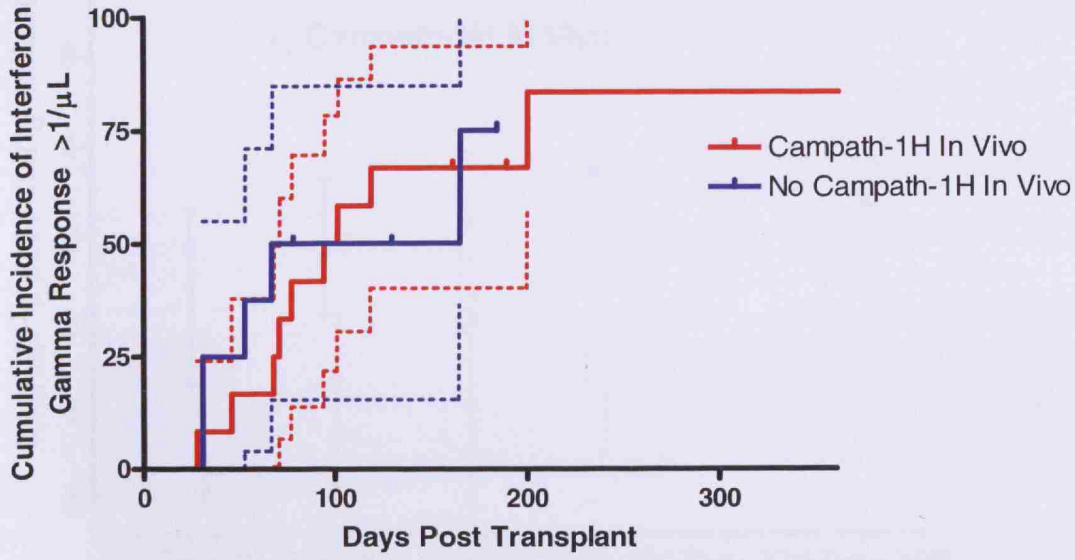


Figure 4-16 CD4+ T response to HCMV according to use of Campath-1H in vivo.

The log_{10} CD4+ T cell interferon gamma response to stimulation with HCMV viral lysate is shown. The results of patients that received Campath-1H in vivo (n=12) are plotted in red, and for patients that did not receive Campath-1H in vivo (n=8) are plotted in blue. The triangles/square boxes represent the median values and the error bars show the interquartile range.

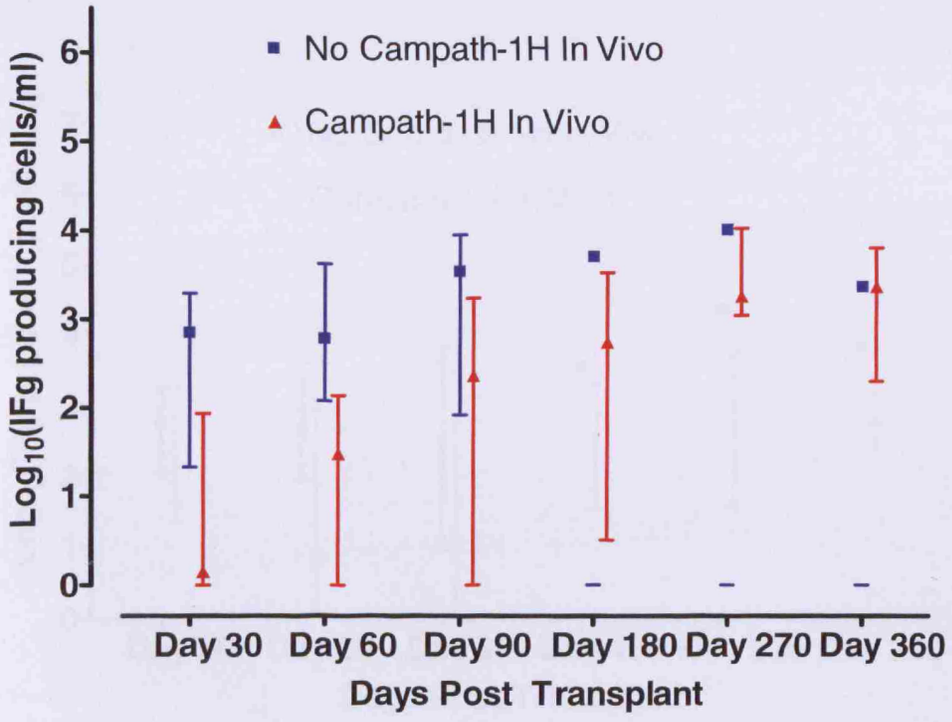
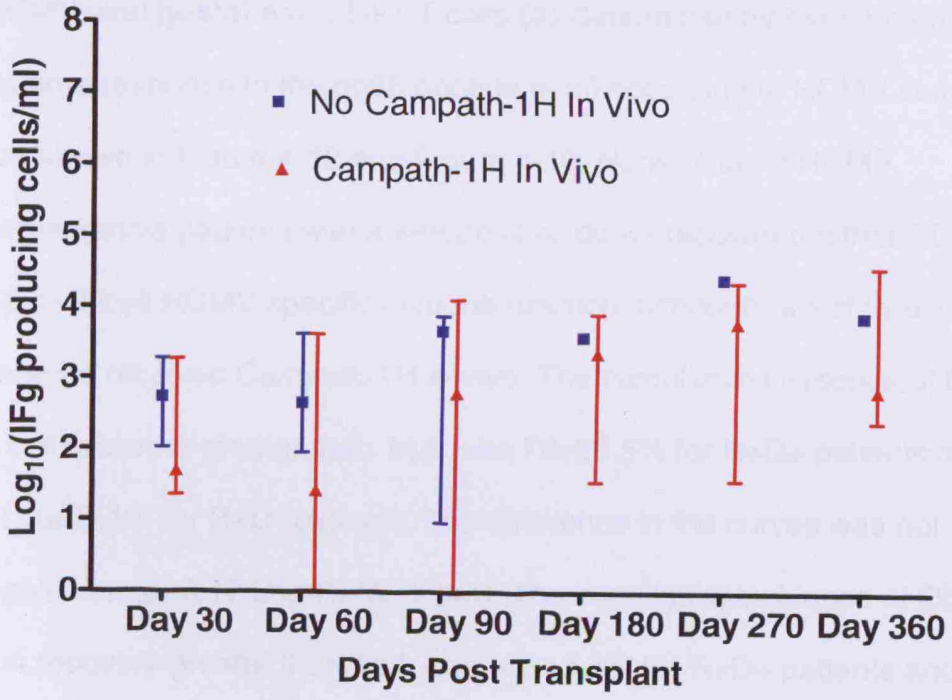


Figure 4-17 CD8+ T Response to HCMV according to use of Campath in vivo.

The log_{10} CD8+ T cell interferon gamma response to stimulation with the pp65 peptide pool is shown. The results of patients that received Campath-1H in vivo (n=12) are plotted in red, and for patients that did not receive Campath-1H in vivo (n=8) are plotted in blue. The triangles/square boxes represent the median values and the error bars show the interquartile range.



4.3.7 Effect of Donor Serostatus on HCMV Immune Recovery

Kaplan Meier survival curves of the cumulative incidence of CD4+ T cell recovery to HCMV (as determined by the interferon gamma response to HCMV viral lysate) and CD8+ T cells (as determined by the interferon gamma response to the pp65 peptide pool) according to HCMV serology are shown in Figure 4-18 and Figure 4-19. None of the 3 HCMV seronegative patients with a seropositive donor recovered either CD4+ or CD8+ T cell HCMV specific immune function, although two of these patients received Campath-1H in vivo. The cumulative incidence of CD4+ T cell recovery greater than 1/μL was 76±28.5% for R+D+ patients and 61.9±39.1% for R+D- patients. The difference in the curves was not significant (p=0.17 Log Rank Score). The cumulative incidence of CD8+ T cell recovery greater than 1/μL was 90±18.6% for R+D+ patients and 81±32.8% for R+D- patients. The difference in the curves was significant (p=0.02 Log Rank Score), with earlier recovery of CD8+ T cell HCMV immunity in patients with a seropositive donor.

Figure 4-18 Kaplan Meier estimate of the cumulative incidence of CD4+ T cell immune recovery to HCMV according to the HCMV serology of the donor and recipient.

The results of the Kaplan Meier estimate of the cumulative incidence of HCMV viral lysate CD4+ T cell interferon gamma response greater than 1/μL according id plotted. The cumulative incidence for R+D+ (n=10, blue line) is 76±28.5%, for R+D- (n=7, red line) is 61.9±39.1%, and for R-D+ (n=3, green line) is 0%. The hatched lines indicate 95% confidence interval. The p value for the log rank score is 0.17, indicating no significant difference between the curves.

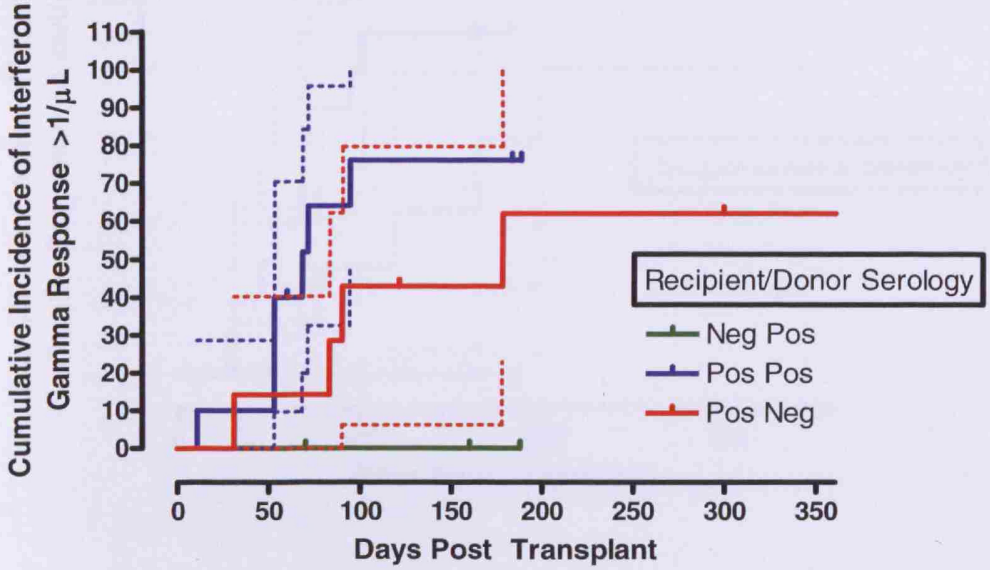
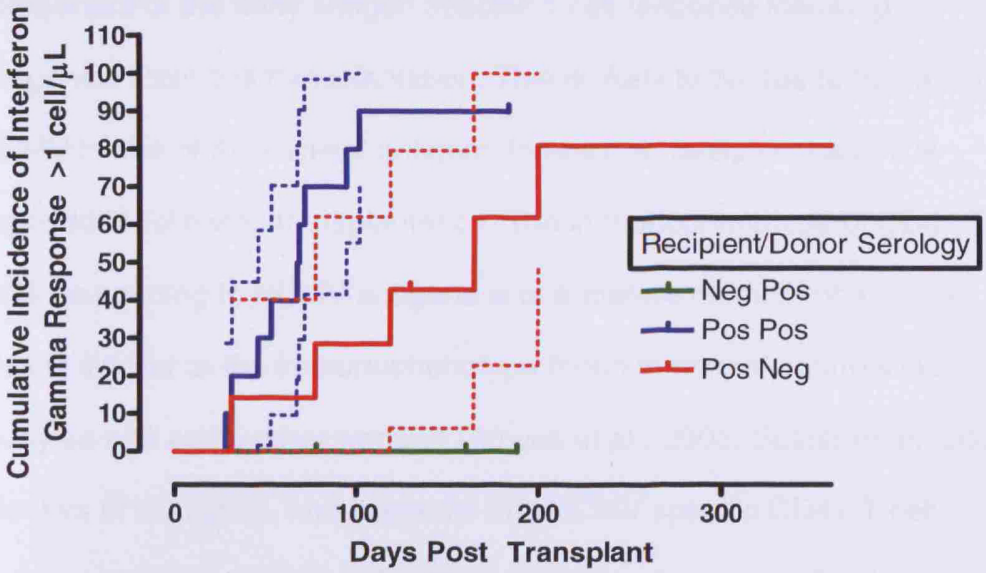


Figure 4-19 Kaplan Meier plot of the cumulative incidence of CD8+ T cell immune recovery to HCMV according to the HCMV serology of the donor and recipient.

The results of the Kaplan Meier estimate of the cumulative incidence of HCMV viral lysate CD4+ T cell interferon gamma response greater than 1/μL according id plotted. The cumulative incidence for R+D+ (n=10, blue line) is 90±18.6%, for R+D- (n=7, red line) is 81±32.8%, and for R-D+ (n=3, green line) is 0%. The hatched lines indicate 95% confidence interval. The p value for the log rank score is 0.02 indicating that the curves are significantly different.



4.4 Conclusions

The HCMV specific CD4+ T cell immune response constitutes a large component of the early antigen specific T cell response following allogeneic stem cell transplantation. This is likely to be due to the fact that HCMV is one of the earliest antigens to which a transplant patient is exposed to following transplantation. The immunophenotype of CD4+ T cells responding to HCMV antigens is of a mature memory phenotype. This is similiar to the immunophenotype found in normal controls in this study as well as by other workers (Amyes et al., 2003; Sester et al., 2002; Weekes et al., 2004), and suggests that HCMV specific CD4+ T cell immune recovery is likely to be derived from mature donor T cells. Overall, 84.6 percent of patients achieved a significant CD8+ T cell response to pp65, with half the patients doing so by 3 months post transplant, similar to the finding in Reussers study (Reusser et al., 1991). However, only 57 percent of patients achieved a significant CD4+ T cell response to HCMV viral lysate, although this difference was not statistically significant due to insufficient study numbers. Even by one year, the absolute CD4+ T cell response did not attain similar levels to normal controls, despite the HCMV specific CD8+ T cell response exceeding normal control levels. This suggests that CD4+ T helper function may be a major limiting factor in the recovery of an HCMV specific immune response following allogeneic stem cell transplant. A low absolute CD4+ T cell count less than 100 X 10^6 has been reported by

Hakki et al. to be associated with delayed recovery of HCMV specific CD4+ T cell function (Hakki et al., 2003).

In this study, an HCMV specific CD4+ T cell response greater than 1 cell/μL was associated with protection against viraemia in all but one patient, while a protective level of HCMV specific CD8+ T cell response could not be established. One possible explanation is that antigen experienced mature HCMV specific CD8+ T cells (derived from the donor) may proliferate in response to exposure to an HCMV antigen, perhaps at a subclinical level, but without HCMV specific CD4+ T helper function, be insufficient to control viral replication and prevent clinically detectable infection. This situation may have parallels to the observation of immunosenescence in HCMV seropositive elderly patients, where high levels of HCMV tetramer positive CD8+ T cells are associated with a terminally differentiated phenotype and functional impairment (Khan et al., 2002b; Ouyang et al., 2003). Although only one case of HCMV disease was observed in this cohort of patients, it is likely that CD4+ T cell function is also essential to prevent HCMV disease. Further work to assess the functional capacity of HCMV specific CD8+ T cells following transplantation is warranted.

The lack of recovery of an HCMV specific immune response in seronegative recipients with a seropositive donor may be to the absence of antigenic stimulation of mature donor derived T cells. Alternatively, as there is evidence that early T cell recovery in patients T-cell depleted with Campath-1 has a significant recipient component (Roux et al., 1996), it may be the absence of HCMV experienced recipient T-cell recovery that

accounts for this. Recovery of HCMV specific CD8+ T cells was significantly faster in seropositive recipients of seropositive donors when compared to seronegative donors. Although recovery of HCMV specific CD4+ T cells was also faster with a seropositive donor, this difference did not reach statistical significance, although that may be due to small study numbers, and the impact of Campath-1H use.

The use of Campath-1H in vivo however was associated with a significantly delayed recovery of HCMV specific CD4+ T cells, although the overall likelihood of recovery of HCMV specific CD4+ T cell function was not affected. No difference was seen in the recovery of HCMV specific CD8+ T cell function. A recent study of 38 patients transplanted with a reduced intensity conditioning regimen using alemtuzumab showed that reconstitution of HCMV specific CTLs as measured by tetramers was rapid, with a five fold rise in HCMV specific CTLs observed following HCMV infection (Lamba et al., 2005). The difference in recovery of HCMV specific CD4+ T helper function is likely to be a significant factor in explaining the high incidence of HCMV infection seen with the use of Campath-1H both in this study as well as other studies (Chakrabarti et al., 2002a).

Recovery of HCMV specific CD4+ T cells is predictive of a low risk of further HCMV infection. Although monitoring of CD4+ T cell function two weekly post transplant is labour intensive, restricting analysis to Day 50 and Day 100 may be a useful way of identifying patients that remain at risk of HCMV infection, and require continued monitoring for HCMV viraemia, as well as identifying potential candidates for immunotherapy.

Chapter 5 Identification of HLA Class II Restricted Epitopes for the HCMV Proteins pp65 and IE1

5.1 Introduction

The identification of immunodominant HLA class II restricted epitopes for the HCMV proteins pp65 and IE1 could provide antigenic peptides to assist in the monitoring of HCMV specific CD4+ T cell function and may facilitate the generation of HCMV reactive cells for immunotherapy. Using the Vβ typing of HCMV specific CD4 cells, it has been demonstrated that there is a clonotypic structure to the CD4 memory response to HCMV, with 1-3 clones predominating, as well as a cohort of subdominant clones and numerous minor clones (Bitmansour et al., 2001). By molecular clonotypic analysis and limiting dilution assays, these clones have been shown to be stable over time and may comprise as much as 0.3 – 1.5% of all CD4+ T cells (Weekes et al., 2004). A similar process of clonal focusing is exhibited by HCMV specific CD8+ T cells (Weekes et al., 1999). Identification of the epitope specificity of these clones may be useful to facilitate immune function monitoring and also have therapeutic potential in the generation of HCMV specific cell lines and peptide vaccines (Paston et al., 2004).

The first HCMV Class I epitopes were identified by generating cytotoxic clones and demonstrating peptide specific cytotoxic activity (Diamond et al., 1997). Using intracellular cytokine staining combined with flow cytometry following antigen stimulation ex vivo, the antigenic potential of a large numbers of peptides can be screened (Kern et al., 1998), and this

approach has already been used to identify Class I epitopes as well as a number of Class II epitopes for pp65 and IE1 (Kern et al., 1999; Kern et al., 2002). As the specificity of an epitope for a particular HLA molecule is determined by anchoring residues within the peptide chain, it is possible to predict Class I specific epitope on the basis of the binding affinity of each possible peptide within a protein using computer algorithms such as SYFPEITHI (Rammensee et al., 1999). Because proteosome cleavage and TAP transport also influences the selection of peptides generated for binding to HLA class I molecules, algorithms have been refined to incorporate these factors into the T-cell epitope prediction algorithms (Doytchinova and Flower, 2006; Donnes and Kohlbacher, 2005; Larsen et al., 2005; Reche and Reinherz, 2005).

With respect to HLA Class II molecules, because of the more relaxed nature of epitope binding and the influence of flanking sequences (Fleckenstein et al., 1999), epitope prediction algorithms are less useful, although they continue to be refined (Cui et al., 2007; Salomon and Flower, 2006; Xiao and Segal, 2005; Nielsen et al., 2004; Tong et al., 2006). Khattab generated pp65 specific CD4+ T cell lines to identify two HLA Class II restricted epitopes to DR11 and DR3 (Khattab et al., 1997). As already discussed, Kern has identified a small number of HLA Class II epitopes to pp65 using overlapping peptides and intracellular cytokine staining (Kern et al., 2002). Weekes identified 3 HLA class-II restricted epitopes by sequencing the TCR beta-chain of peptide specific CD4+ T cell clones (Weekes et al., 2004). Other authors have described epitopes for gB (Elkington et al., 2004) and pp150 (La Rosa et al., 2005), while

only one Class II restricted epitope to IE1 has been described to date (Gautier et al., 1996). Recently Li Pira reported the results of a pp65 specific Class II peptide mapping study using immunomagnetic CD4+ T cell selection and ELIspot (Li Pira et al., 2004a). A summary of the Class II HCMV specific epitopes identified to date is shown in Table 5-1. The feasibility of using overlapping peptide pools to generate CTL for immunotherapy has been evaluated and this technique is also capable of generating CD4 responses (Trivedi et al., 2005) and has been used to generate CTLs. With respect to the generation of HCMV specific cell lines for the use in immunotherapy, clinical trials in the past have utilised either autologous fibroblasts infected with HCMV AD169 or whole HCMV AD169 protein derived from an infected fibroblast cell line. However the problem is that CTLs derived from this method may not reflect the same epitope response that is seen in vivo and thus may not be fully effective.

A CD4 helper response to HCMV using proliferation assays has been described for pp65, gB, gH, IE1, IE2, and UL69 (Beninga et al., 1995), with the response to pp65 and IE1 being among the most frequent. Individual epitopes to gB (Elkington et al., 2004; Liu et al., 1993) , gH (Elkington et al., 2004), IE1 (Alp et al., 1991; Gautier et al., 1996) and pp65 (Bitmansour et al., 2001; Gallot et al., 2001; Kern et al., 2002; Khattab et al., 1997; Trivedi et al., 2005; Weekes et al., 2004; Davignon et al., 1996; Li Pira et al., 2004b) have been described..

Table 5-1 HLA Class II HCMV specific epitopes previously published for pp65 and IE1.

Protein	*Epitope site*	*Sequence*	*HLA serologic*	*HLA molecular*	*Ref*
pp65	361 to 376	PQYSEHPTFTSQYRIQ	DR11	DRB1*11	(Khattab et al., 1997)
pp65	485 to 499	PPWQAGILARNLVPMV	?DR3	DRB1*03	(Khattab et al., 1997)
pp65	509 to 524	KYQEFFWDANDIYRIF	DR3	DRB1*03	(Khattab et al., 1997)
pp65	364 to 386	SEHPTFTTSQYRIQGKLEYHTWD	DR1302	DRB1*1302	(Gallot et al., 2001)
pp65	34 to 56	VLPHETRLLQTGIHVRVSQPSLI	DQ0602	DQB1*0602	(Gallot et al., 2001)
pp65	364 to 386	SEHPTFTTSQYRIQGKLEYHTWD	DR11	DRB1*11	(Gallot et al., 2001)
pp65	34 to 56	VLPHETRLLQTGIHVRVSQPSLI	DQ0602	DQB1*0602	(Gallot et al., 2001)
pp65	509 to 523		?		(Bitmansour et al., 2001)
pp65	511 to 522		?		(Bitmansour et al., 2001)
pp65	47 to 58		?		(Bitmansour et al., 2001)
pp65	41 to 55	LLQTGIHVRVSQPSL	DR15 (DQ6)	DRB1*15	(Kern et al., 2002)
pp65	281 to 299	IIKPGKISHIMLDVAFTSH	DR53 (DQ3)	DRB4*01	(Kern et al., 2002)
pp65	361 to 383	PQYSEHPTFTSQYRIQGKLEYRH	DR11	DRB1*11	(Kern et al., 2002)
pp65	509 to 527	KYQEFFWDANDIYRIFAEL	DR52	DRB3*01 or 02	(Kern et al., 2002)
pp65	489 to 507	AGILARNLVPMVATVQGQN	?		(Kern et al., 2002)
pp65	41 TO 60	LLQTGIHVRVSQPSLILVSQ	DQ6	DQB1*06	(Weekes et al., 2004)
pp65	259 to 273	NPQPFMRPHERNGFT	DR13	DRB1*13	(Weekes et al., 2004)
pp65	367 to 380	PTFTDQYRIQGKLE	DQ11	DQB1*06	(Weekes et al., 2004)
pp65	41 to 55	LLQTGIHVRVSQPSL	DR15	DRB1*15	(Li Pira et al., 2004b)
pp65	117 to 131	PLKMLNIPSINVHHY	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	117 to 131	PLKMLNIPSINVHHY	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	169 to 183	TRQQNQWKEPDVYYT	DR1	DRB1*01	(Li Pira et al., 2004b)
pp65	177 to 191	EPDVYYTSAFVFPTK	DR7	DRB1*07	(Li Pira et al., 2004b)
pp65	225 to 239	KVYLESFCEDVPSGK	DR15	DRB1*15	(Li Pira et al., 2004b)
pp65	245 to 259	TLGSDVEEDLTMTRN	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	261 to 275	QPFMRPHERNGFTVL	DR13	DRB1*13	(Li Pira et al., 2004b)
pp65	281 to 295	IIKPGKISHIMLDVA	DR4	DRB1*04	(Li Pira et al., 2004b)
pp65	281 to 295	IIKPGKISHIMLDVA	DR7	DRB1*07	(Li Pira et al., 2004b)
pp65	365 to 379	EHPTFTSQYRIQGKL	DR11	DRB1*11	(Li Pira et al., 2004b)
pp65	373 to 387	YRIQGKLEYRHTWDR	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	413 to 427	TERKTPRVTGGGAMA	DR14	DRB1*14	(Li Pira et al., 2004b)
pp65	429 to 443	ASTSAGRKRKSASSA	DR11	DRB1*11	(Li Pira et al., 2004b)
pp65	445 to 459	ACTSGVMTRGRLKAE	DR1	DRB1*01	(Li Pira et al., 2004b)
pp65	489 to 503	AGILARNLVPMVATV	DR11	DRB1*11	(Li Pira et al., 2004b)
pp65	489 to 503	AGILARNLVPMVATV	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	509 to 523	KYQEFFWDANDIYRIF	DR1	DRB1*01	(Li Pira et al., 2004b)
pp65	509 to 523	KYQEFFWDANDIYRIF	DR3	DRB1*03	(Li Pira et al., 2004b)
pp65	513 to 523	FFWDANDIYRI	DR1301	DRB*1301	(Trivedi et al., 2005)
pp65	513 to 523	FFWDANDIYRI	DQ05xx	DQB1*05	(Trivedi et al., 2005)
pp65	513 to 523	EHPTFTSQYRIQGKL	DR1101	DRB1*1101	(Trivedi et al., 2005)
IE1	162 to 175	DKREMWMACIKELH	DR8		(Gautier et al., 1996)

5.2 Methods

5.2.1 Patient Population

Normal controls were recruited from healthy HCMV seropositive laboratory workers. Ten HCMV seropositive stem cell transplant recipients were recruited from the HCMV specific immune reconstitution study described in Chapter 4, including nine allogeneic stem cell transplant recipients and one autologous transplant recipient. Only patients who had recovered sufficiently both an absolute CD4+ T cell count and HCMV specific CD4+ T cell responses were included in the study of HLA Class II HCMV specific epitopes. The details of the patient recruited and sample processing are described in sections 4.2.1 and 4.2.2

5.2.2 Antigen Stimulation and Intracelluiar Cytokine Staining

The method for mononuclear cell preparation is described in section 4.2.6. The method for antigen stimulation in section 4.2.7 and for intracellular cytokine staining is described in section 4.2.8. The antigens used for the Class II epitope identification study were HCMV AD169 viral lysate (ABI, Columbia, USA) at a concentration of 5 µg per 1ml of cell suspension (containing $1X10^6$ PBMCs), pp65 and IE1 peptide pools (Jerini Peptide Technologies, Germany) at a concentration of 1µg per 1ml ($1X10^6$ PBMCs) of cell suspension, 1µg of each peptide mapping pool for IE1 and pp65 (Jerini Peptide Technologies, Germany) per 1ml ($1X10^6$ PBMCs) of cell suspension for low resolution mapping, and 1 µg of each

individual 15 mer peptide suspension (Alta Biosciences, Birmingham, UK) for high resolution mapping.

The low resolution mapping pools used for identifying candidate sites for immunodominant Class II epitopes each contained 15 to 17 consecutive 15 mer peptides, each peptide sequence overlapping the preceding peptide sequence by 11 amino acids. Eight peptide pools spanning the entire sequence of IE1 and 9 pools spanning the entire sequence of pp65 were used. The composition of the low resolution mapping pools is detailed in Appendix B. For high resolution epitope mapping, all the individual 15 mer peptides from a reactive pool were tested individually. All peptides were manufactured by Fmoc solid phase peptide synthesis.

Monoclonal antibodies (BD Biosciences, Oxford, UK) used for low resolution mapping were CD4 FITC, CD3 PerCP, CD8 PE (surface staining) and Interferon γ APC (intracellular staining). For high resolution mapping, the monoclonal antibodies CD4 FITC, CD3 PerCP, CD69 PE (surface staining) and Interferon Gamma APC (intracellular staining) were used.

HCMV specific CD4+ T cell responses were screened using HCMV viral lysate, and IE1 and pp65 peptide pools and the low resolution mapping pools for IE1 and pp65. In order to identify the peptide sequence of the immunodominant epitope, a second PBMC sample from patients reacting to a low resolution mapping pool was stimulated with the individual constituent peptides of that pool in order to identify the immunodominant 15 mer peptide chain.

5.2.3 FACS Analysis

Following surface and intracellular staining, the fixed cell suspensions were acquired using a FACSCalibur four colour flow cytometer (BD Bioscience, Oxford, UK). FACS analysis was performed using CellQuest software (BD Bioscience, Oxford, UK). Initial gating to identify the lymphocyte population was performed using forward and side scatter. A secondary gate was placed around the CD3+ CD4+ population to identify CD4+ T Cells. The threshold fluorescence intensity for interferon γ APC positive cells was set using unstimulated control cells. The same gating strategy was used for the identification of CD4+ T cells for high resolution mapping. Interferon γ reactivity was confirmed by the demonstration of upregulation of the activation marker, CD69 on an Interferon γ versus CD69 plot, with reactivity calculated by quadrant analysis.

5.2.4 Epitope Prediction Modelling

In order to define the HLA Class II restriction of immunodominant regions identified by high resolution, the amino acid sequence was entered into the epitope prediction program, SYFPEITHI, maintained by Prof. Hans-Georg Rammensee at the Department of Immunology, University of Tuebingen, Germany, and accessed by the website www.syfpeithi.de (Rammensee et al., 1999). Epitope prediction is only available for the HLA Class II molecules: DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, and DRB1*1501. Anchoring and binding residues are predicted from previously published motifs. The program performs a sequential analysis of each 15 mer peptide of the entered sequence, and

assigns a score based on the presence of ideal anchors, auxiliary anchors and preferred residues, with negative values for amino acids excluded at a particular site for the selected HLA molecule. The maximum score predicts the most likely epitope. Because the program calculates epitope binding affinity for 15 mer peptides and the binding groove contains 9 residues, the first anchoring residue is the fourth amino acid of the peptide.

5.2.5 HLA Typing

HLA typing was performed by the Anthony Nolan Trust. Low resolution Class I and II HLA typing was performed by serology and high resolution typing was performed by PCR using sequence specific oligonucleotides.

5.3 Results

5.3.1 HLA Typing

A total of 22 normal controls and 10 stem cell transplant recipients were studied. The HLA Class I and II tissue types for normal controls are shown in Table 5-2 and for the stem cell recipients in Table 5-3.

Table 5-2 HLA class I and II molecular typing results of normal controls.

(Results in italics indicate serological typing)

Code	*Gender*	*HLA Class I A and B*	*Class II HLA DRB1*	*Class II HLA DQB1*
C1	Female	A1, A1, B7, B8	0301, 0401	
C2	Female	A2, A2, B15, B39	0401, 1101	
C3	Female	A11, A30, B13, B58	0301, 0701	
C4	Male	A1, A24, B15, B27	1301	
C5	Male	A26, B38, B27	0401, 1301	
C6	Male	A2, B15	*4, 13*	06*
C7	Male	A32, A33, B52, B58	1502, 1302	
C8	Female	A1, A68, B35, B51	1101, 1301	0603, 0301
C9	Male	A24, A31, B56	*11, 15*	
C10	Female	A1, A26, B8, B55	0301, 1401	
C11	Female	A3, A11, B35, B42	1501, 0801	06*, 03*
C12	Male	A24, A35, B35, B44	16*, 13*,	
C13	Female	A2, A29, B27, B44	0101, 1101	0501, 0301
C14	Female	A2, A29, B7, B44	1501	06*
C15	Male	A2, A11, B7, B40	1501	*1*
C16	Female	A1, A3, B35, B37	0101, 1501	
C17	Male	A1, A2, B35	1303, 1401	0301, 0503
C18	Female	A1, A2, B15, B38	0301	0201
C19	Female	A11, A68, B35	0405, 0805	
C20	Female	A2, A3, B7, B18	1501, 0401	0302, 0602
C21	Female	A2, A24, B7, B35	*2, 4*	
C22	Female	A24, A68, B15, B27	0101, 1103	0501, 0301

* unresolved at allele level

Table 5-3 HLA class I and II molecular typing results of stem cell transplant patients.

Code	*Gender*	*HLA Class I A and B*	*Class II HLA DRB1*	*Class II HLA DQB1*
P1	Female	A24, A31, B51, B27	0401, 0408	03*, 03*
P2	Male	A1, A2, B7	0103, 1501	0501, 0602
P3	Female	A2, A32, B51	1104, 1301	0603, 0301
P4	Male	A29, A68, B49, B50	0102, 1001	0501
P5	Male	A2, A31, B50,B35	1501, 0701	06*, 02*
P6	Male	A2, A2, B50, B35	1104, 1001	0501, 03*
P7	Female	A24, A11, B15, B35	0101, 1103	0501, 03*
P8	Female	A24, A11, B51, B27	1202, 0912	03*, 0303
P9	Female	A1, B8	1107, 0701	02*, 03*
P10	Female	A1, A2, B8, B40	0101, 03011	0501, 02*

* unresolved at allele level

5.3.2 Results of HCMV Lysate and Peptide Pool Responses

The patient and control CD4+ T cell IFγ responses to HCMV AD169 viral lysate, pp65 and IE1 peptide pools are shown in Table 5-4. The percentage of the total CD4+ T cells of the patient population responding to HCMV viral lysate (1.34%) and the IE1 peptide pool (0.06%) significantly exceeded the percentage of CD4+ T cell response of the normal controls (0.45% for HCMV viral lysate and 0.03% for the IE1 peptide pool, p = 0.01 and 0.03 respectively, Mann Whitney U test). The difference in the percentage response to the pp65 peptide pool between the patients and the controls did not reach statistical significance (0.41% for the patient population and 0.21% for the normal controls, p=0.11 Mann Whitney U test). The percentage pp65 response of the normal controls contributed to just less than half the percentage HCMV viral lysate response.

For normal controls, the absolute response to the HCMV viral lysate was 3.65 IFγ producing cells per μL, with the median absolute response to the pp65 peptide pool (1.97 IFγ producing cells per μL) accounting for just over half the viral lysate response, while the median IE1 response (0.27 IFγ producing cells per μL) accounted for seven percent of the viral lysate response. For the post transplant patient population, the absolute HCMV viral lysate response was 3.99 IFγ producing cells per μL, with the pp65 peptide pool response (0.81 IFγ producing cells per μL) accounting for twenty percent of the viral lysate response, and the IE1 peptide pool response (0.24 IFγ producing cells per μL) accounting for six percent.

There was no significant difference in the absolute IFγ response for any of the antigens.

Table 5-4 CD4+ T cell interferon gamma response of patients and controls to HCMV viral lysate, and the pp65 and IE1 peptide pools.

Code	*% HCMV Lysate Response*	*Absolute Lysate Response (IFγ/μl)*	*% IE1 response*	*Absolute IE1 response (IFγ/μl)*	*% pp65 response*	*Absolute pp65 Response (IFγ/μl)*
C1	0.93	7.12	0.03	0.26	0.28	2.17
C2	0.21	1.84	0.01	0.12	0.12	1.04
C4	0.41	2.56	0.00	0.00	0.00	0.00
C5	0.45	3.22	0.08	0.59	0.38	2.75
C6	0.77	5.98	0.07	0.51	0.25	1.96
C7	0.27	2.04	0.03	0.26	0.04	0.29
C8	1.81	20.49				
C9	0.03	0.11	0.01	0.05	0.05	0.22
C10	0.53	4.78	0.11	0.97	0.41	3.73
C11	0.19	2.49				
C12	0.22	1.22	0.05	0.28	0.07	0.38
C13	0.55	3.65	0.02	0.11	0.30	1.98
C14	1.23	12.28	0.03	0.29	0.51	5.08
C15	0.55	6.01	0.03	0.38	0.19	2.01
C16	0.97	14.91	0.00	0.05	0.19	2.89
C17	0.54	4.11	0.08	0.60	0.23	1.77
C18	0.06	0.47	0.03	0.27	0.03	0.27
C20	0.08	0.72				
C22	0.38	5.77	0.00	0.06	0.22	3.34
Median Control	*0.45*	*3.65*	*0.03*	*0.27*	*0.21*	*1.97*
P1	0.22	0.20	0.01	0.01	0.04	0.03
P2	6.59	7.59	0.06	0.07	0.69	0.80
P3	1.13	3.82	0.06	0.20	0.66	2.23
P4	2.09	3.99	0.55	1.05	0.22	0.42
P5						
P6	1.34	7.09	0.03	0.14	0.15	0.81
P7	0.64	2.74	0.14	0.58	0.41	1.77
P8	6.83	14.53	1.03	2.18	5.06	10.76
P9	9.26	35.54	0.84	3.23	2.12	8.13
P10	0.26	1.19	0.05	0.24	0.10	0.43
Median Patients	*1.34*	*3.99*	*0.06*	*0.24*	*0.41*	*0.81*
P value *Mann Whitney*	*0.01*	0.52	*0.03*	0.52	*0.11*	0.95

Lysate and peptide data for C19 and C21 unavailable

5.3.3 Low Resolution Mapping

Low resolution mapping was performed on all 32 individuals in the study. Representative plots of the FACS analysis of a normal control response to pp65 and IE1 low resolution mapping pools is shown in Figure 5-1 and Figure 5-2. The percentage of CD4+ T cells producing interferon γ in response to stimulation with the pp65 mapping pools is shown in Figure 5-3 and for IE1 mapping pool is shown in Figure 5-4. Applying a threshold response of 0.05%, 30 of the 32 (94 percent) individuals in the study had a response to at least one pp65 peptide pool as illustrated in Figure 5-5. The median response was to two pp65 peptide pools (range 0 to 7), and although all the peptide pools elicited at least one response, the most immunogenic regions of pp65 were identified as pools 1 (aa 1 to 71), 4-7 (181 to 431) and pool 9 (aa 481 to 561). Twenty-one out of 32 (55%) individuals responded to at least one peptide pool from IE1, with 9 out of 10 (90%) patients having a response, Figure 5-6. The median response was to 1 peptide pool, with a range of 0 to 7. The most immunogenic regions of IE1 were pools 1 (aa 1 to 71) and 6 (aa 301 to 371), although the patient cohort also showed a significant response to pool 4 (aa 181 to 251) and pool 5 (aa 241 to 311).

Patients were significantly more likely to respond to a greater number of pp65 and IE1 mapping pools than the normal controls, with a median response of 3.5 pools for both pp65 and IE1 compared to a median response of only 1 pool for pp65 and IE1 for the controls ($p=0.03$ for pp65 and $p=0.04$ for IE1, Mann Whitney U Test), Figure 5-7. Thus the CD4+

immune response of transplant recipients is characterised by greater diversity than normal controls. As the total CD4+ response as characterised by the response to HCMV lysate is similar between the two groups, the magnitude of the individual epitope responses in transplant recipients is likely to be lower.

Figure 5-1 Representative FACS plots of the CD4+ T Cell interferon gamma response to pp65 low resolution mapping pools.

The interferon γ response of CD4+ T cells following stimulation with the pp65 low resolution mapping pools is shown for the normal control, C14. IFγ APC vesus FSC is plotted for CD4+CD3+ cells for each mapping pool. The percentage positive cells are defined as the percentage of cells in the right upper quadrant.

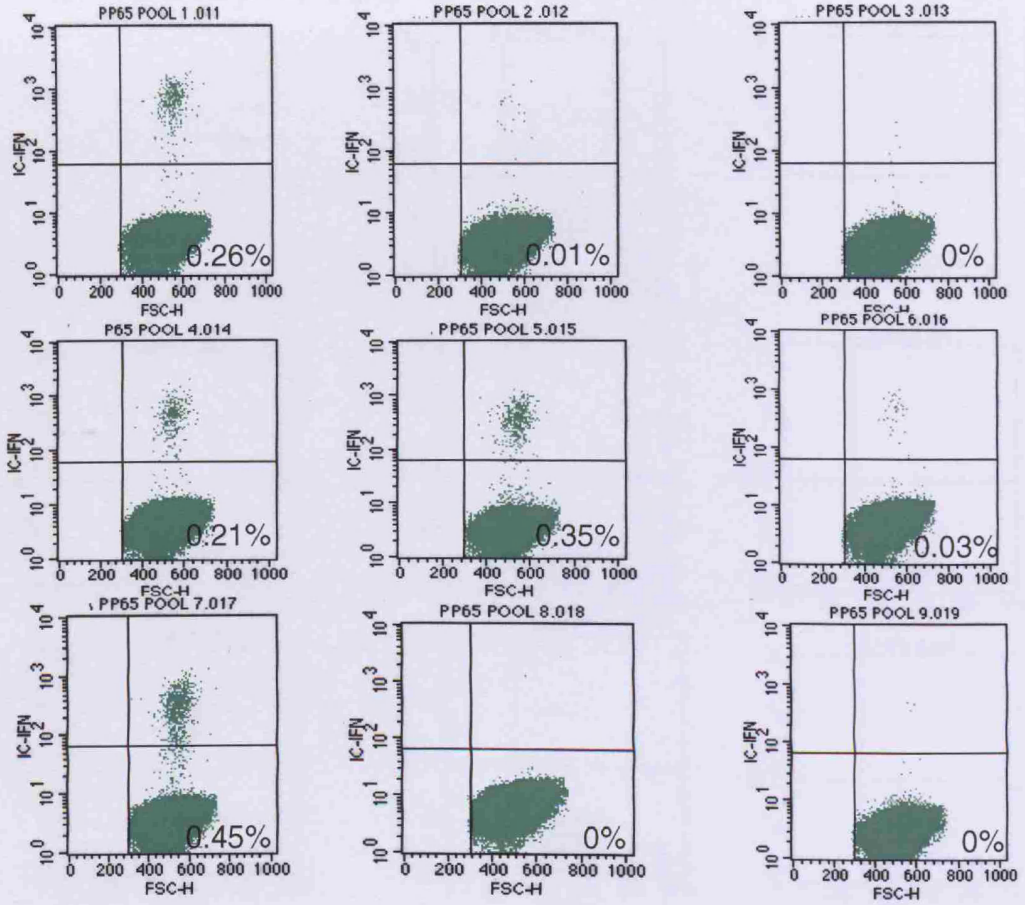


Figure 5-2 Representative FACS plots of the CD4+ T Cell interferon gamma response to IE1 low resolution mapping pools.

The interferon γ response of CD4+ T cells following stimulation with the IE1 low resolution mapping pools is shown for the normal control, C14. IFγ APC vesus FSC is plotted for CD4+CD3+ cells for each mapping pool. The percentage positive cells are defined as the percentage of cells in the right upper quadrant.

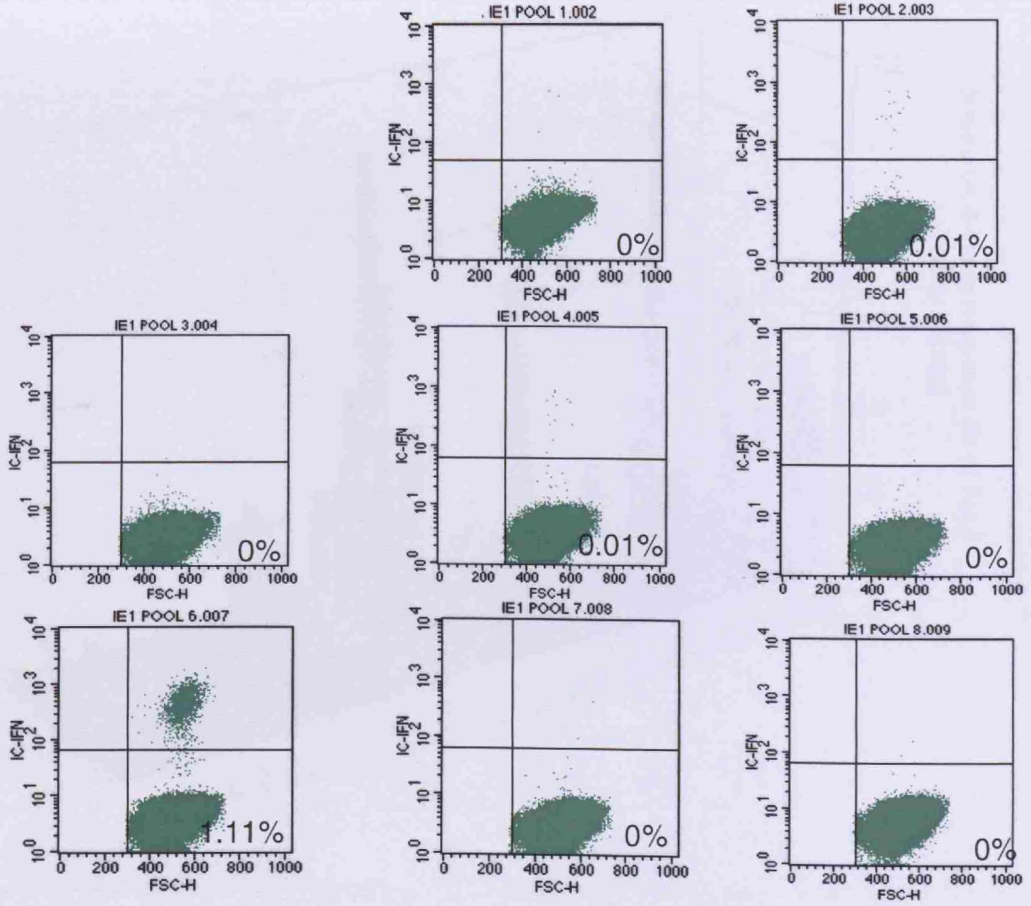


Figure 5-3 Results of low resolution pp65 mapping of normal controls and patients.

The results of low resolution mapping of normal controls (n=21) and patients (n=10) with the pp65 low resolution mapping pools is illustrated as a three dimensional bar graph. The results are expressed as the percentage of the total CD4+ T cells producing interferon gamma in response to stimulation with each of the nine mapping pools.

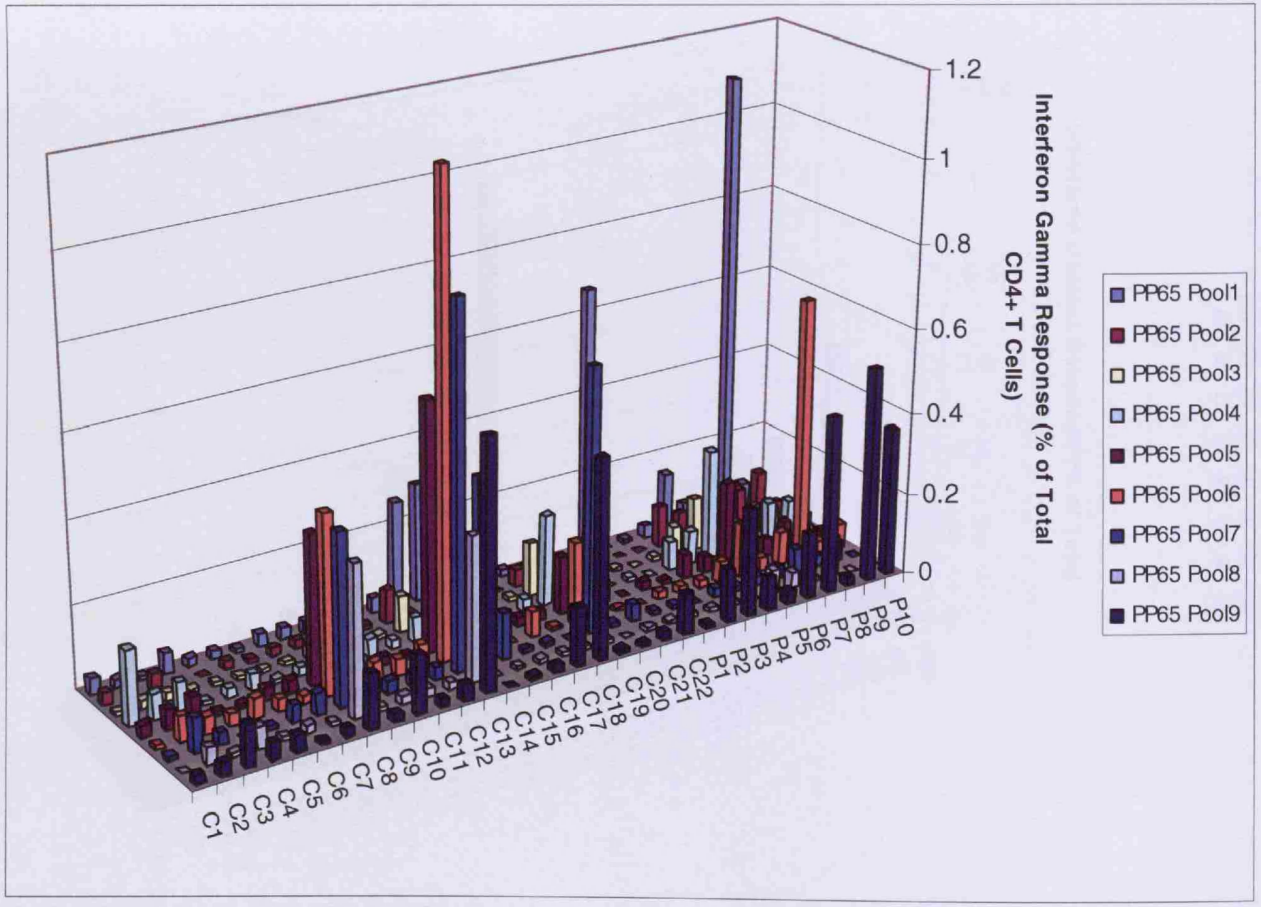


Figure 5-4 Results of low resolution IE1 mapping of controls and patients.

The results of low resolution mapping of normal controls (n=21) and patients (n=10) with IE1 low resolution mapping pools is illustrated as a three dimensional bar graph. The results are expressed as the percentage of the total CD4+ T cells producing interferon gamma in response to stimulation with each of the eight mapping pools.

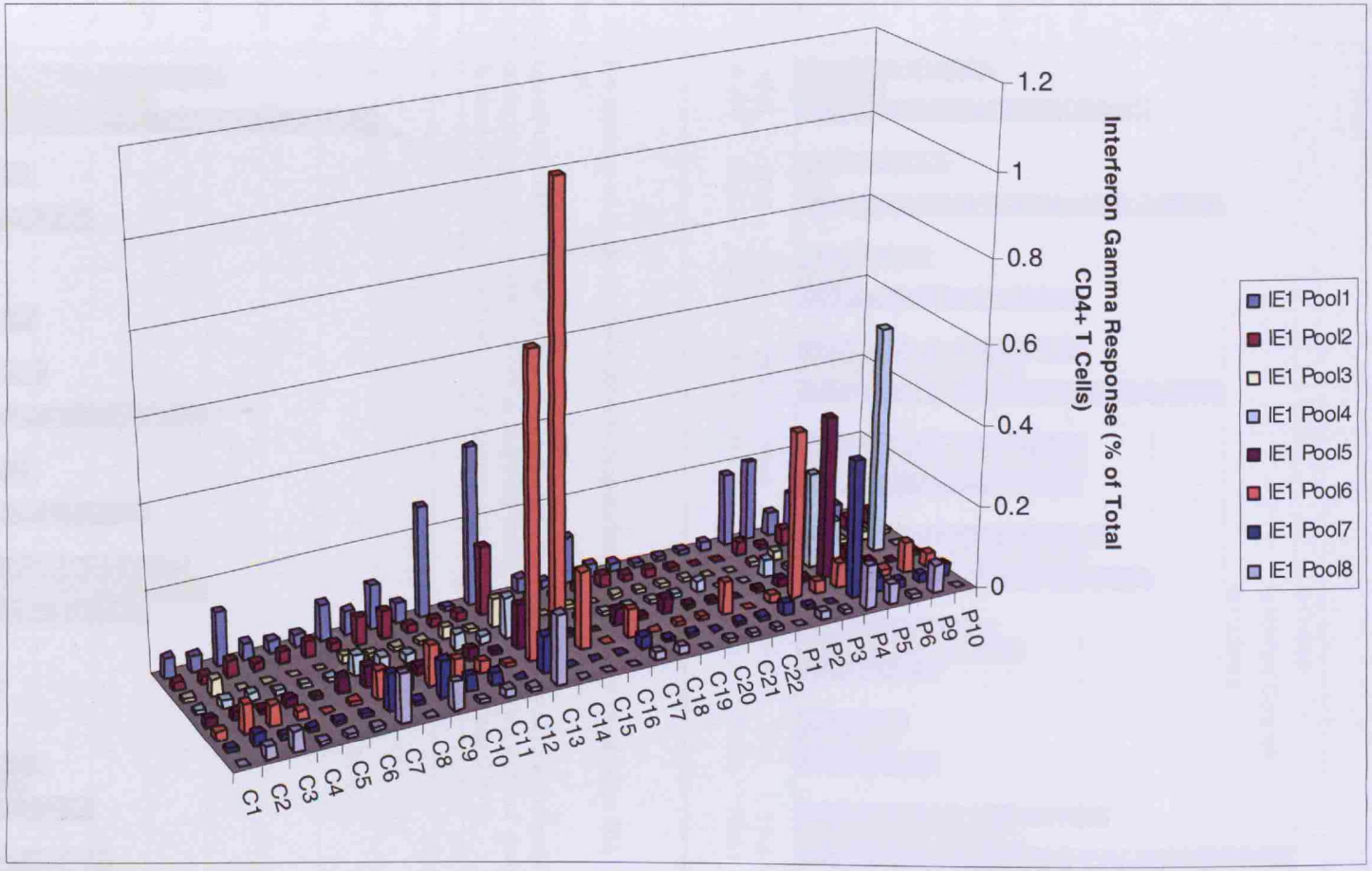


Figure 5-5 Percentage of patients and controls responding to each low resolution pp65 mapping pool.

The percentage of the overall group (green, n=32), normal controls (red, n=22) and patients (blue, n=10) responding to each of the low resolution mapping pools of pp65 is shown as a bar graph. A response is defined as a CD4+ T cell interferon gamma response of greater than 0.05%.

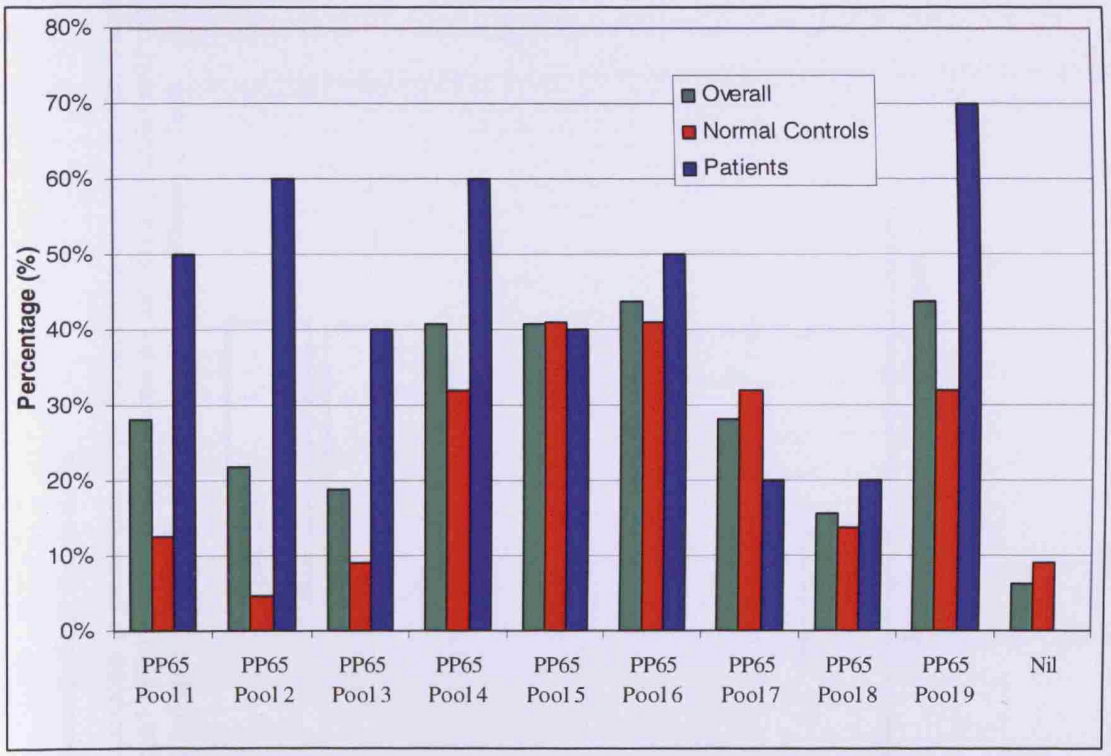


Figure 5-6 Percentage of patients and controls responding to each low resolution IE1 mapping pool.

The percentage of the overall group (green, n=32), normal controls (red, n=22) and patients (blue, n=10) responding to each of the low resolution mapping pools of IE1 is shown as a bar graph. A response is defined as a CD4+ T cell interferon gamma response of greater than 0.05%.

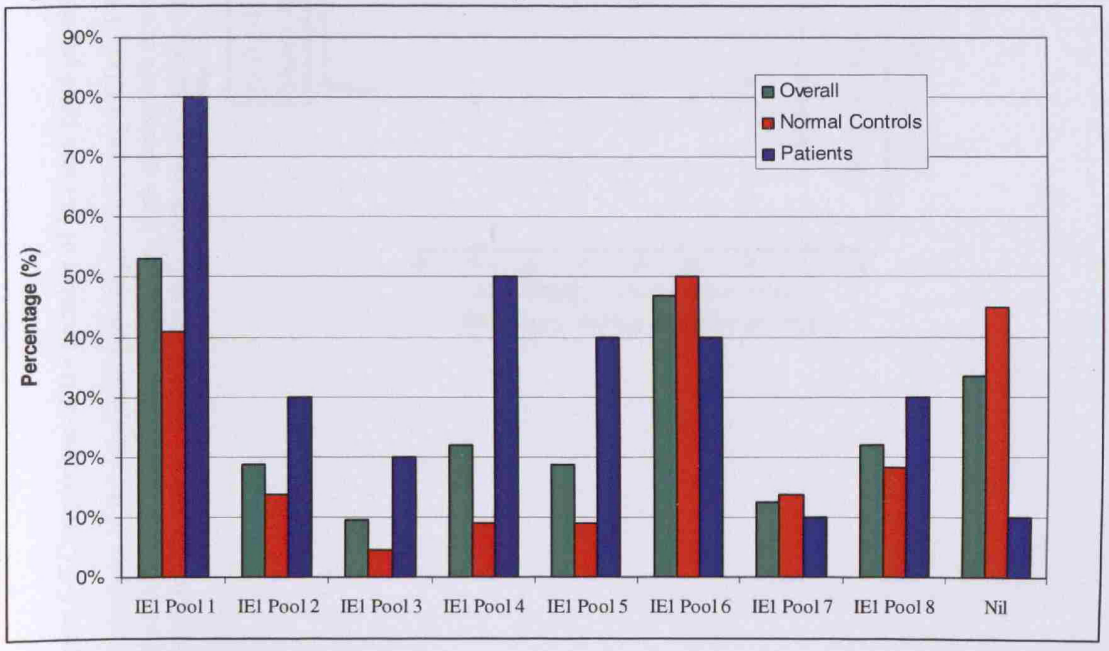
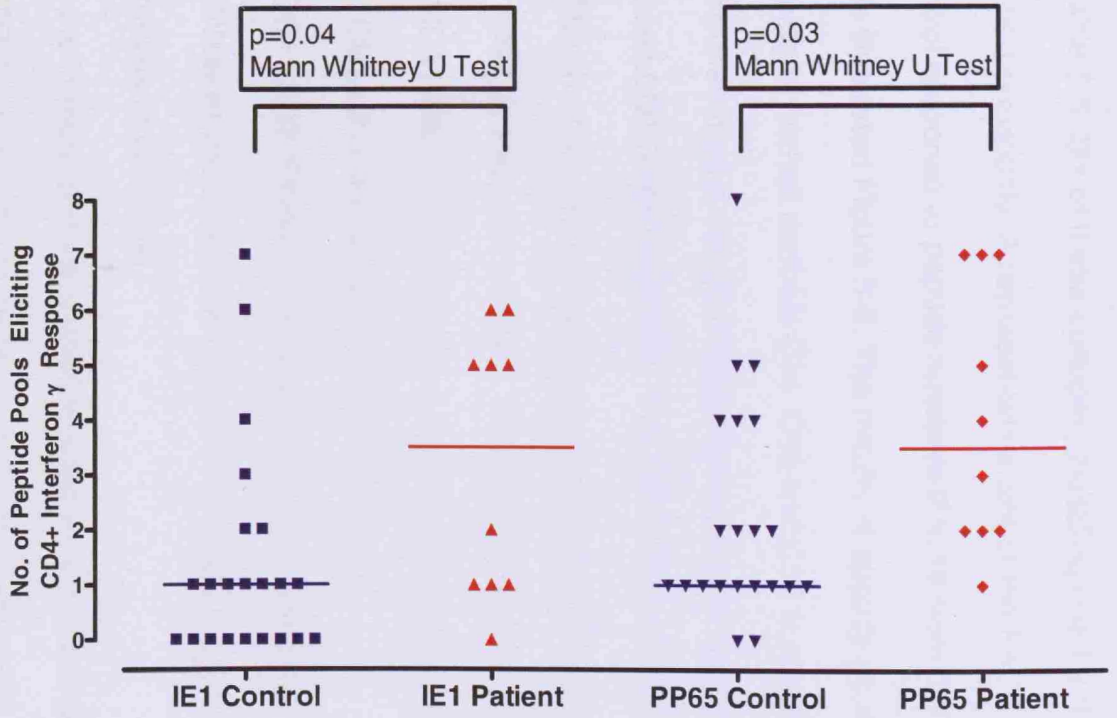


Figure 5-7 Comparison of the number of IE1 and pp65 peptide pool responses between patients and controls.

A scatter plot of the number of peptide pools eliciting a CD4+ T Cell interferon gamma response of greater than 0.05% for patients and normal controls is shown. The horizontal line indicates the median value. Each blue square represents the number of pp65 or IE1 pools eliciting a response for the normal controls (n=22) and the red triangle for the patients (n=10). The difference was statistically significant for both IE1 and pp65 (p=0.04 and 0.03 respectively, Man Whitney U Test)



5.3.4 High Resolution Mapping

High-resolution mapping was performed in 15 individuals (9 normal controls and 6 patients) to define HLA Class II restricted pp65 and IE1 epitopes. High-resolution mapping identified thirteen individual HLA Class II epitopes, and they are shown along with their likely HLA Class II associations, Table 5.5. Six of these epitopes, including one for IE1 have not been described previously. A representative plot of the FACS analysis of a normal control response to peptide numbers 9 to 12 from the pp65 peptide pool 1 is illustrated Figure 5-8. The results of peptide pool 1 high resolution mapping of normal controls C14, C15 and C16 is shown in Figure 5-10, demonstrating a maximal response to peptide number 11, with the sequence LLQTGIHVRVSQPSL. This epitope has previously been described by Khattab and Li Pira, but ascribed to HLA DR15 (Khattab et al., 1997; Li Pira et al., 2004b). Although all three responding individuals are HLA DRB1*15, based on predicted anchoring residues the most likely HLA Class II restriction for this epitope is HLA DQA1*0103/DQB1*0602 (Ettinger and Kwok, 1998), Figure 5-10, consistent with Weekes data demonstrating an HLA DQ6 restriction for this epitope (Weekes et al., 2004).

For patient P1, the immunodominant pp65 epitope was found to be HVLKAVFSRGDTPVLPHETRLLQ (pp65 aa 21 to 33), with equal responses to peptides number 7, 8, and 9 of pp65 pool 1. The most likely HLA restriction is HLADRB1*0401 as predicted by anchoring residues previously defined for this Class II molecule (Friede et al., 1996), with F in position 1 of the nonamer binding groove. When this sequence was

entered into the SYFPEITHI epitope prediction program for HLA DRB1*0401, the 15 mer peptide, KAVFSRGDTPVLPHE achieved the maximum binding score of 22, Table 5-5.

For control C13, the epitope does seem likely to be DRB1*1101, and corresponds with the predicted anchoring residues with F in position 1 of the binding groove (Verreck et al., 1996), and the predicted epitope from SYFPEITHI with a score of 26 is **HPTFTSQYR**IQGKLWE (auxiliary and main anchoring residues in bold), and this concurs with Li Pira findings.

The epitope YRHTWDRHDEGAAQG does not appear to be an HLA DRB1*1501 epitope as the binding score following SYFPEITHI analysis is only 4. A DQB1*06 restriction may be possible but as this epitope was only identified in one individual, this cannot be confirmed.

The epitope PPWQAGILARNLVPMVATV (pp65 aa 485 to 503) appears to be restricted to either DRB1*1101 or DQB1*0301. Interestingly La Pira suggested that the epitope could be presented by either DRB1*11 or DRB1*0301 but an alternative explanation is that the epitope is in fact presented by a DQB1 molecule shared by these individuals, as La Pira does not report the results of HLA Class II DQ typing in her patient population. Analysis of the epitope by SYFPEITHI support a non-DRB1 restriction for this epitope, as the binding score for DRB1*0301 is 13 (Table 5-7) and DRB1*1101 is 15 (Table 5-8).

It appears likely that the IE1 epitope, MESSAKRKMDPDNPD (aa 1 to 15) is HLA DQB1*06 restricted, although this is based on only two individuals.

It was not possible to attribute a likely association to the pp65 epitopes SVLGPISGHVLKAVFSRGD (aa 13 to 31), VALRHVVCAHELVCS (aa

193 to 207), YRHTWDRHDEGAAQG (aa 381 to 395), TERKTPRVTGGGAMA (aa 413 to 427), GQNLKYQEFFWDANIYRI (aa 517 to 531) and ANDIYRIFAELEGVW (aa 517 to 531).

Table 5-5 HLA class II epitopes identified at high resolution.

(results in italics indicate serological typing)

Protein	*Site*	*Sequence*	*Code*	*HLA DR*	*HLA Association*	*Previously Described*
IE1	1 to 15	MESSAKRKMDPDNPD	C17	DR13,14 DQ3,5	DQ5	No
IE1	1 to 15	MESSAKRKMDPDNPD	P2	DR1,15 DQ5,6	DQ5	No
pp65	13 to 31	SVLGPISGHVLKAVFSRGD	P8	DR9,12 DQ3		No
pp65	21 to 43	HVLKAVFSRGDTPVLPHETRLLQ	P1	DR4 DQ3	DR4	No
pp65	41 to 55	LLQTGIHVRVSQPSL	C14	DR15 DQ6	DQ6	*(Li Pira et al., 2004b) DR15,* (Kern et al., 2002) *DR15,* (Weekes et al., 2004) *DQ6*
pp65	41 to 55	LLQTGIHVRVSQPSL	C15	DR15 *DQ1(6)*	DQ6	
pp65	41 to 55	LLQTGIHVRVSQPSL	C16	DR1,15	DQ6	
pp65	193 to 207	VALRHVVCAHELVCS	C1	DR3,4		No
pp65	361 to 383	PQYSEHPTFTSQYRIQGKLEYRH	C13	DR1,11 DQ3,5	DR11	(Li Pira et al., 2004b) *DR11*
pp65	381 to 395	YRHTWDRHDEGAAQG	C14	DR15 DQ6		No
pp65	413 to 427	TERKTPRVTGGGAMA	C13	DR1,11 DQ3,5		(Li Pira et al., 2004b) *DR14*
pp65	485 to 503	PPWQAGILARNLVPMVATV	C13	DR1, 11	DR11 or DQ3	(Li Pira et al., 2004b) *DR11 or DR3*
pp65	485 to 503	PPWQAGILARNLVPMVATV	C22	DR1,11 DQ3,5	DR11 or DQ3	
pp65	485 to 503	PPWQAGILARNLVPMVATV	P3	DR11,13 DQ3,6	DR11 or DQ3	
pp65	485 to 503	PPWQAGILARNLVPMVATV	P6	DR10,11 DQ3,5	DR11 or DQ3	
pp65	485 to 503	PPWQAGILARNLVPMVATV	P7	DR1,11 DQ3,5	DR11 or DQ3	
pp65	489 to 503	AGILARNLVPMVATV	C18	DR3 DQ2	DR3	(Li Pira et al., 2004b) *DR11 or DR3,* (Khattab et al., 1997) *?DR3,* (Kern et al., 2002) *Not ascribed*
pp65	505 to 523	GQNLKYQEFFWDANIYRI	P6	DR10,11 DQ3,5		(Li Pira et al., 2004b) *DR1 or DR3*
pp65	505 to 523	GQNLKYQEFFWDANIYRI	P7	DR1,11 DQ3,5		
pp65	505 to 527	GQNLKYQEFFWDANIYRIFAEL	C22	DR1,11 DQ3,5		
pp65	509 to 523	KYQEFFWDANDIYRI	C18	DR3	DR3	(Li Pira et al., 2004b) *DR3 & DR1,* (Trivedi et al., 2005) *DQ5,* (Kern et al., 2002) *DR52,* (Bitmansour et al., 2001) *Not Ascribed*
pp65	509 to 531	KYQEFFWDANDIYRIFAELEGVW	C10	DR3,14 DQ3,4		
pp65	517 to 531	ANDIYRIFAELEGVW	P6	DR10,11 DQ3,5		(Trivedi et al., 2005) *DR11*
pp65	517 to 531	ANDIYRIFAELEGVW	P7	DR1,10 DQ3,5		

Table 5-6 SYFPEITHI epitope prediction for HLA DRB1*0401 for the pp65 immunodominant sequence, HVLKAVFSRGDTPVLPHETRLLQ (aa 21 to 33).

Position of 1st Peptide	*Peptide Number* 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	*score*
4	**K** A V **F** S **R** **G** D T P V L P H E	22
1	**H** V L **K** A V **F** S **R** G D T P V L	18
8	S R G **D** T **P** V L P H E T R L L	12
9	**R** G D T P V L P **H** E T R L L Q	12
3	**L** K A V F S **R** G **D** T P V L P H	9
5	A V F S **R** **G** **D** T **P** V L P H E T	6
2	V L K **A** V **F** S **R** G D T P V L P	0
6	V F S **R** G **D** T **P** V L P H E T R	0
7	F S R **G** D T **P** V **L** P H E T R L	0

(Anchoring residues in bold)

Table 5-7 SYFPEITHI epitope prediction for HLA DRB1*0301 for the pp65 immunodominant sequence, PPWQAGILARNLPMVATV (aa 485 to 503).

Position of 1st Peptide	*Peptide Number* 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
4	Q A G **I** L A R N **L** V P M V A T	13
5	A G I **L** A **R** N L **V** P M V A T V	11
1	P P W Q A **G** I L **A** R N L V P M	10
3	W Q A **G** I L A R **N** L V P M V A	9
2	P W Q A **G** I L A **R** N L V P M V	8

(Anchoring residues in bold)

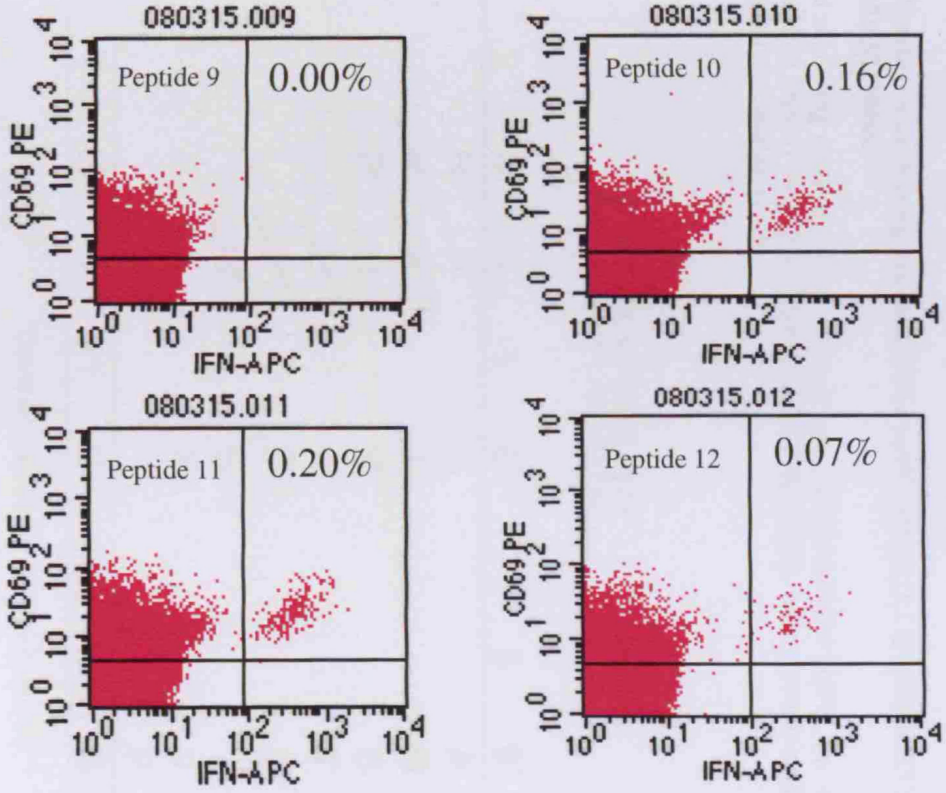
Table 5-8 SYFPEITHI epitope prediction for HLA DRB1*1101 for the pp65 immunodominant sequence, PPWQAGILARNLVPMVATV (aa 485 to 503).

Position of 1st Peptide	*Peptide Number* 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
2	P W Q **A** G I L **A** R N L V P M V	15
1	P P W Q A G I L A R N L V P M	7
3	W Q A **G** I L A R N L V P M V A	7
4	Q A G I L A R N L V P M V A T	6
5	A G I L A **R** N L **V** P M V A T V	6

(Anchoring residues in bold)

Figure 5-8 Representative FACS plots of high resolution peptide mapping for the identification of an immunogenic epitope from pp65 peptide pool 1.

Representative FACS plots of the CD4+ T cell response to high resolution mapping for the normal control C15 are shown, illustrating the response to stimulation with the individual mapping peptides 9 through to 12 from pp65 mapping pool 1. The interferon γ response is defined as the percentage of cells in the right upper quadrant.



Peptide No.	pp65 Pool 1
1	MESRGRRCPEMISVL
2	GRRCPEMISVLGPIS
3	PEMISVLGPISGHVL
4	SVLGPISGHVLKAVF
5	PISGHVLKAVFSRGD
6	HVLKAVFSRGDTPVL
7	AVFSRGDTPVLPHET
8	RGDTPVLPHETRLLQ
9	PVLPHETRLLQTGIH
10	HETRLLQTGIHVRVS
11	LLQTGIHVRVSQPSL
12	GIHVRVSQPSLILVS
13	RVSQPSLILVSQYTP
14	PSLILVSQYTPDSTP
15	LVSQYTPDSTPCHRG

Figure 5-9 Interferon gamma response as a percentage of total CD4+ T Cells following stimulation with individual peptides from pp65 peptide pool 1.

A three dimensional bar graph of the percentage of CD4+ T cells producing interferon γ in response to the individual mapping peptides from pp65 pool 1 is shown for normal controls C14 (brown), C15 (blue), and C16 (green). The maximal response is to peptide 11 (aa 41 to 55) with the sequence LLQTGIHVRVSQPSL.

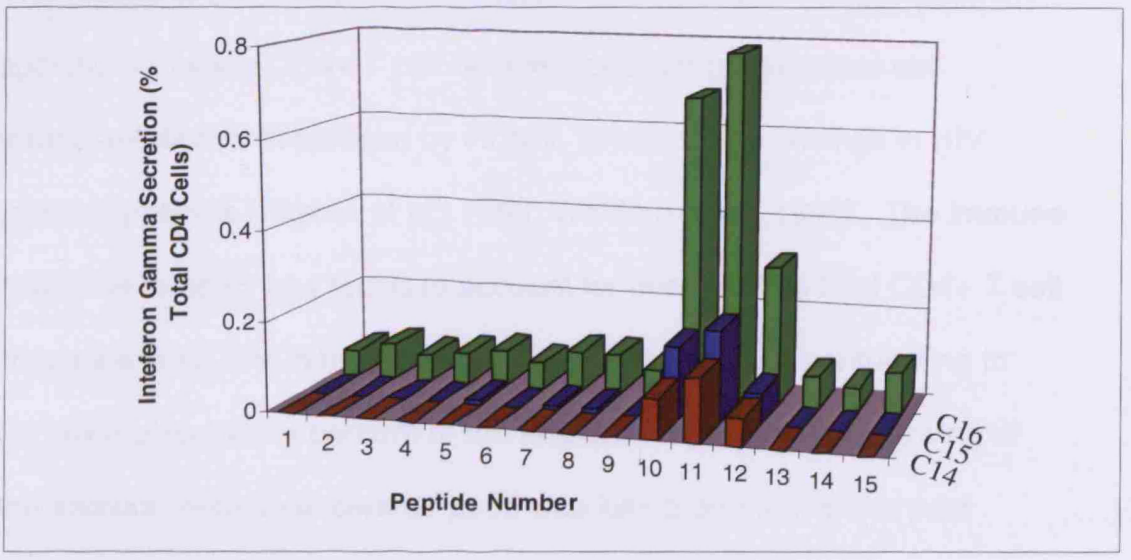
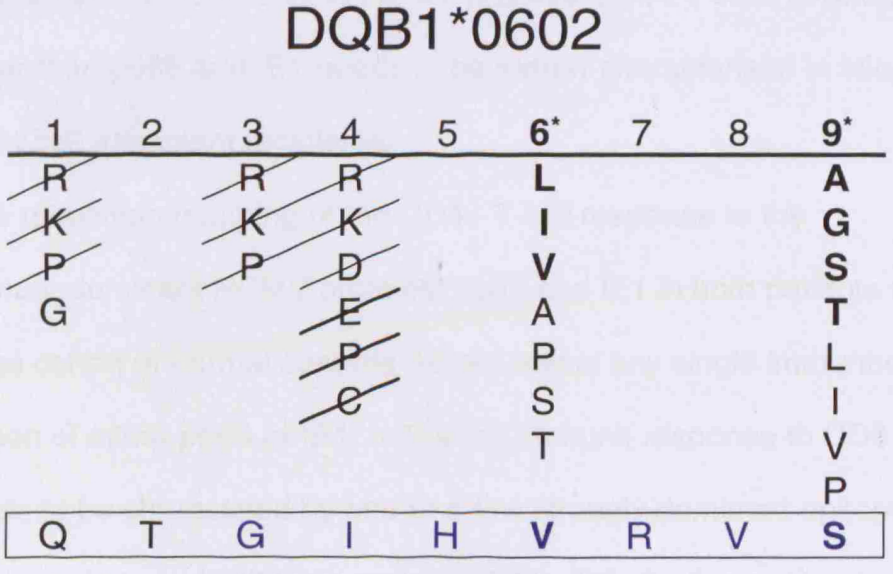


Figure 5-10 Predicted anchoring residues of the pp65 epitope, LLQTGIHVRVSQPSL for DQA1*0103/DQB1*0602.

The predicted anchoring residues for the pp65 epitope, LLQTGIHVRVSQPSL (aa 45 to 55) is shown for the HLA class II molecule, DQA1*0103/DQB1*0602, with glutamine (Q) occupying the 1st position of the binding groove.



5.4 Conclusions

The results of this study confirm that in HCMV seropositive patients, the specificity of early CD4+ T cell recovery post allogeneic stem cell transplantation is dominated by HCMV, similar to the findings in HIV infected patients (Pitcher et al., 1999: Waldrup et al., 1997). The immune response to pp65 was found to account for over half the total CD4+ T cell response to HCMV in healthy normal controls, with IE1 contributing to approximately seven percent of the response. However the skewing of the immune response towards pp65 was less prominent in the post transplant patients, accounting for only twenty percent of the response to HCMV viral lysate. Some of this difference is due to a proportionately greater immune response to IE1, but this study suggests that other antigens, such as gB and pp150 may also make significant to the immune response. The specificity of HCMV reactive CD4+ T cells to antigens other than pp65 and IE1 needs to be further characterised in allogeneic stem cell transplant recipients.

Low resolution mapping of the CD4+ T cell response to the immunodominant HCMV proteins, pp65 and IE1 in both patients and a large cohort of normal controls did not reveal any single immunodominant region of either pp65 or IE1, unlike the immune response to CD8 which tends to be charactised by one or a few strongly dominant epitopes (Diamond et al., 19997; Kern et al., 1999). This finding contrasts with those of Bitmansour (Bitmansour et al., 2001) who found that the CD4+ T cell response to HCMV was dominanted by 1 to 3 clones. However this

work was done using T cell lines generated following prolonged stimulation with HCMV antigen in vitro, whereas by assessing the functional response of unexpanded T cells dircectly from the patient, this study is more likely to reflect the true nature of the HCMV specific immune response in vivo. Among stem cell transplantation recipients, the immune response was characterised by a broader range of epitopes than the normal controls, suggesting that the immune response is less focused. Weekes found in normal controls by clonotypic analysis that the CD4+ T cell immune response to HCMV showed a focused but stable pattern over time (Weekes et al., 2004). Thus the broader range of responses seen in the allogeneic transplant recipient may represent facets of the early immune response to HCMV. Alternatively, reactivation of HCMV in the allogeneic transplant setting may increase the frequency of HCMV specific subdominant CD4+ T cell clones. It is not clear if a broader antigenic response with multiple epitopes is required to control HCMV infection in the stem cell transplant setting or if a response to a few dominant epitopes are sufficient. Analysis of HCMV specific C8+ T cell responses in HIV patients using overlapping peptides suggest that patients with a broader antigenic repertoire are better able to control replication and prevent disease, despite having a similar absolute response (Sacre et al., 2005). In healthy individuals, there is evidence that in order to mount and sustain a functional immune response to HCMV, both a diversity in antigen specificity and a persistent CD4+ T cell response are required for protective immunity (Sinclair et al., 2004). A comprehensive assessment of the CD4+ T cell immune response to

HCMV in a larger number of allogeneic stem cell transplant recipients over a longer time period and correlation with HCMV viral load data is required to clarify this question. This issue also has important practical implications as restricting the range of HCMV reactive clonotypes when generating HCMV specific CD4+ T cells line for immunotherapy, such as using peptide pools from a single protein such as pp65 (Rauser et al., 2004) may compromise efficacy. In contrast, strategies that generate HCMV specific CD4+ T cells with a broad range of specificities such as through the use of HCMV infected autologous fibroblasts or whole viral lysate (Walter et al., 1995; Peggs et al., 2001) would overcome this issue. This data also suggests that monitoring immune reconstitution by HCMV specific class II tetramers will have significant limitations due to the broad antigenic repertoire observed in stem cell transplant recipients and the low frequency of individual epitopes.

In this chapter, high resolution mapping was successfully used to identify immunodominant epitopes in both normal controls and patients. However, the attribution of class II HLA restrictions to the epitopes identified proved more problematic. Despite the poor specificity in empirically predicting Class II epitopes, prediction algorithms such as SYFPEITHI (Rammensee et al., 1999) were useful in attributing an immunodominant peptide sequence to an HLA DRB1 molecule based on predicted binding motifs, as well as helping to to narrow down the most likely sequence. However, for HLA DR there is only a single alpha chain allele, so all the polymorphisms are determined by the beta chain, simplifying epitope prediction for DRB1. For HLA DP and DQ, both the alpha and the beta

chain are highly polymorphic and both contribute to the peptide binding specificity. The association of DRB2, 4 and 5 alleles in association with certain DRB1 types adds another level of complexity to the problem of defining class II epitopes.

In a number of cases, the results of HLA Class II association of the identified epitopes disagreed with previously published HLA associations (Kern et al., 2002; Weekes et al., 2004; Li Pira et al., 2004b). One explanation is that some of the authors, including Li Pira, did not consider peptide presentation through HLA DQB1 (Li Pira et al., 2004b). All three HLA class II families (DP, DQ and DR) have previously been shown to be important for CD4+ helper T cell responses (Gehrz et al., 1987). The results of my study support the importance of DQ in HCMV antigen presentation. Presentation through DQ may account for the apparent ability of an epitope to be presented by more than one DRB1 allele suggested by other workers (Burrows et al., 2003). Compared to DR, there are only a limited number of DQ alleles which tend to be relatively conserved among different populations. It is possible that infection with HCMV has exerted selective pressure in the spectrum of DQ alleles over time, as HCMV is known to have co-evolved with humans (McGeoch et al., 1995). Further work is required to confirm which HLA Class II family (DP, DQ or DR) an epitope is restricted to by performing blocking experiments using monoclonal antibodies which block the Class II binding site at the time of incubation of PBMCs with the target peptide.

In a recent study using overlapping peptides extending across the entire HCMV genome, at least 151 ORFs containing immunogenic peptide

sequences for both CD8+ and CD4+ T-cells have been described (Sylwester et al., 2005). As the investigators used peptides overlapping by 10 amino acids, they were not able to accurate define all epitopes but did find prominent responses to both the IE1 and pp65 ORFs. However, these workers also showed in order to obtain a comparable CD4+ T cell response to a peptide pool spanning the entire HCMV genome, peptides from a minimum of 6 of the most immunogenic ORFs need to be used. Therefore, restricting antigen responses to only pp65 and IE1 may limit the immune response generated in some individuals, and the immunogenicity of additional HCMV proteins needs to be studied, for possible inclusion as a source of antigenic stimuli for immunotherapy.

As a result of the findings from this study, thymidine proliferation assays should be performed to confirm that the identified epitopes are capable of stimulating CD4+ T cell proliferation. Furher work is needed to define the clonotypic nature of the responding CD4+ T cell by analysing Vbeta usage. Following peptide stimulation, Vbeta usage could be assessed either by flow cytometry using fluorescent labelled Vbeta specific monoclonal antibodies (IOTEST Beta Mark kit, Immunotech, Marseille), or alternatively by PCR, using Vbeta specific primer sets following selection of reactive cells by cell sorting or gamma capture technique (Miltenyi). The results could be compared to a clonogram derived following stimulation with the whole HCMV viral lysate to assess for other dominant or subdominant clones. This information would further enhance the understanding of the role of HCMV specific CD4+ T cells in protecting allogeneic stem cell transplant patients from HCMV infection.

Chapter 6 General Discussion

The purpose of this thesis was to increase understanding of HCMV infection and the role of the immune system in controlling HCMV infection in allogeneic stem cell transplantation, in order to find ways of reducing the morbidity and mortality associated with HCMV disease. The risk factors for HCMV infection were identified in a large cohort of allogeneic transplant patients and are described in Chapter Two of this thesis. The ability of HCMV replication kinetics derived from real time PCR monitoring of HCMV viral load to predict HCMV disease and the response to antiviral therapy is described in Chapter Three. A prospective study of the recovery of CD4+ and CD8+ T cell immunity to HCMV following allogeneic stem cell transplantation is described in Chapter Four. A study to characterise the CD4+ T cell response to HCMV and identify class II HLA epitopes for the HCMV proteins pp65 and IE1 in stem cell transplant patients and normal controls is described in Chapter Five.

In the large cohort of patients described in the second chapter of this thesis, neither recipient or donor HCMV serology has a significant impact on survival, and this provides indirect evidence for the efficacy of the combination of high dose prophylactic aciclovir and a pre-emptive strategy to prevent HCMV disease while limiting the adverse consequences of antiviral therapy, such as the increased rates of infection associated with ganciclovir use (Boeckh et al., 1996; Salzberger et al., 1997). The absence of viraemia in the R-D- group supports the approach of HCMV seronegative blood products for this group of patients.

In the era of universal leukodepletion the necessity of this may be disputed, but as there is evidence of greater HCMV disease following transfusion of non-HCMV seronegative leukodepleted red cells (Bowden et al., 1995), this approach should remain. The low incidence of HCMV infection seen in allogeneic transplant recipients with R-D+ HCMV serology, suggests that the acquisition of HCMV infection from a positive donor is not inevitable. Therefore, although these patients are at a higher risk of HCMV infection than R-D- patients, and need HCMV surveillance with pre-emptive therapy for viraemia, they also require HCMV seronegative blood products.

Chapter Two also identified the use of Campath-1 in vivo, but not ex vivo, as a major risk factor for HCMV infection. The impact of in vivo Campath use on HCMV infection was greatest in the R+D- group compared to R+D- patients not receiving Campath in vivo, most likely due to the effect on pre-existing recipient HCMV specific immunity. The finding that HCMV infection occurs significantly earlier in patients receiving Campath-1G or Campath-1H compared to patients not receiving Campath in vivo supports the hypothesis that the use of Campath in vivo has the greatest impact on the recovery of early HCMV specific immune function. The fact that the median time to onset of HCMV infection in patients receiving Campath-1H was 30.5 days and the very long half life of the antibody has important implications for immunotherapy, as the prophylactic infusion of HCMV specific immune competent cells could be neutralised by the anti-CD52 activity of Campath-1H.

This study has also shown that the change from Campath-1G to Campath-1H has resulted in an increase in HCMV infection, and this is most likely due to the longer half life of the humanised form of Campath (Morris et al., 2003). The same in vivo dose was used for both Campath-1G and Campath-1H in the patients described in Chapter Two, therefore patients receiving Campath-1H experienced a far greater level of in vivo T-cell depletion. A reduction in the dose of Campath-1H in vivo may reduce the risks of excessive T-cell depletion such as increased HCMV infection, and such dose de-escalation studies are underway. In a recent study, 30 milligrams of Campath-1H given for three days pre-transplant resulted in significantly delayed recovery of CD4+, CD8+, CD19+ and NK cells compared to ATG (Thymoglobulin 2mg/kg for 3 days), but a reduction of the dose of Campath to a single dose of 30mg was shown to correct this delay without compromising immune suppression (Juliusson et al., 2006). Initial results of the UK Campath-1H de-escalation trial, published in abstract form, have shown that, compared to 60mg of Campath, reduced doses are not associated with increased mortality (Chakraverty et al., 2006). The timing of Campath-1H is also critical, as a greater incidence of GHVD is seen if Campath-1H in vivo is used 7-8 days prior to stem cell transplant compared to 24-48 hours prior to transplant.

Ex vivo use of Campath-1H for T-cell depletion was not associated with a significantly increased risk of HCMV infection in this study, and may represent an alternative approach to reducing graft versus host disease without compromising immune reconstitution (Chakrabarti et al., 2003a;

Novitzky et al., 2004). This approach and its impact on the recovery of HCMV specific immunity warrants further investigation.

The study of risk factors for late HCMV infection described in Chapter 2 is subject to selection bias as only patients perceived to be at risk of HCMV infection underwent extended HCMV PCR surveillance. In this high risk cohort, patients experiencing HCMV infection before 100 days were identified as being at the greatest risk for late HCMV infection on univariable Cox regression analysis. However, in a multivariable analysis, this factor was no longer significant, while increasing recipient age, acute GVHD, and a low CD34 dose were significant. These factors all impair the long term recovery of immune function following allogeneic stem cell transplantation. Use of Campath in vivo was no longer a significant factor after 100 days, suggesting that the greatest impact of Campath is on HCMV infection risk early post transplant. Due to the inherent selection bias of this study, these results must be interpreted with caution and can only be applied to patients clinically perceived to be at high risk of HCMV infection. Nevertheless, older patients, those with a poor stem cell dose and patients experiencing GVHD, as well as patients with HCMV infection and a seronegative donor should all have extended monitoring for HCMV viraemia beyond 100 days.

Real time PCR monitoring of HCMV viral loads can be used to guide pre-emptive therapy, and has the advantage of being a very rapid test, with a turnaround time of only 24 hours compared to the minimum two day turnaround time using agarose gel based PCR techniques. It has been

shown that viral load kinetics provides useful prognostic information, predicting the likely response to therapy and the risk of disease. The peak viral load, a faster viral replication, and a prolonged duration of viraemia were all found to be significant predictors of patients at increased risk of HCMV disease, and these patients need to be targeted for more intensive therapy and monitoring. Although the peak viral load and faster viral replication rates have previously been recognised as risk factors for HCMV disease (Emery et al., 2000; Gor et al., 1998), the results from Chapter Three confirm that they remain significant when a strategy of pre-emptive therapy for HCMV infection is employed. Because the results are available in real time, they can be used for clinical management of patients with HCMV infection. The rate of viral replication can easily be calculated from the first two consecutive PCR positive results or the last negative and the first positive PCR result, and provides the clinician with useful information, if sufficiently frequent viral monitoring, ideally twice weekly is performed. In a multivariable analysis, it was found that the duration of viraemia is the most significant risk factor for HCMV disease, and this is consistent with Regoes' recent finding that the risk of disease has a better correlation with the total viral turnover rather then a single peak viral load (Regoes et al., 2006). Thus, the goal of pre-emptive therapy should be to reduce the total exposure of stem cell transplant patients to HCMV viraemia, rather than simply reducing peak viral loads. A new finding from this study is that a delayed response to therapy, with a peak viral load occurring after initiation of therapy also predicts an increased risk of disease, and this is likely to reflect the more rapid viral

replication rate, probably due to an absence of HCMV specific immunity seen in these patients.

The strategy of implementing HCMV specific antiviral therapy when the viral load rises above a level of 3000 genome copies of HCMV per ml, or two consecutive HCMV PCR positive results, resulted in a substantial number of patients receiving therapy unnecessarily, as evidenced by the absence of viraemia at the time therapy was initiated. Using viral replication rates, these patients can be identified, and further monitoring continued before initiating therapy. The most appropriate threshold for the initiation of therapy, as well as when to discontinue therapy remains to be determined, and further clinical studies to establish this are required.

Viral load dynamics have also demonstrated the equivalence in efficacy of ganciclovir and combination therapy with ganciclovir/foscarnet. The same approach should be used to evaluate the efficacy of new therapies for HCMV, such as valganciclovir.

HCMV viral load kinetics by real time PCR was not able to predict the risk of recurrence of HCMV infection. The only factor able to significantly predict an increased risk of recurrence in patients experiencing a first episode of HCMV infection was the receipt of stem cells from an HCMV seronegative donor. Unfortunately, although these patients are also most likely to benefit from adoptive immunotherapy, this is only possible if they have an HCMV seropositive donor. HCMV vaccination of seronegative donors could be evaluated as an alternative preventative strategy in these patients, although this does raise ethical issues.

The phenotype of HCMV specific CD4+ T cells in patients following allogeneic transplant was found to be of an antigen experienced mature phenotype and similar to the phenotype of HCMV specific CD4+ T cells in the normal controls used in this study and described by others (Amyes et al., 2003; Sester et al., 2002; Weekes et al., 2004). In this study HCMV specific CD4+ T cell recovery as determined by the response to the HCMV viral lysate was found to be a dominant component of early immune recovery post transplant, but absolute levels take at least a year to reach the same levels as normal controls. The response to HCMV viral lysate gave the best indication of the status of HCMV specific immunity in the transplant recipient. pp65 specific responses recover early post transplant, closely mirroring viral lysate responses, while the IE1 recovery was slower. An HCMV specific T cell response of greater than one cell per μL was associated with a low risk of HCMV infection, while no protective level for HCMV specific CD8+ T cells by functional analysis could be demonstrated, in contrast to the findings using HCMV specific CD8+ tetramers in allogeneic stem cell transplant patients (Aubert et al., 2001). Because HCMV infection only occurred in the absence of significant HCMV specific CD4+ T cell function, it was not possible to assess the impact of the level of immunity on the replication rate.

It was found that HCMV specific CD4+ T cell recovery was slower than CD8+ T cell recovery. Patients with HCMV infection had a delayed recovery of HCMV specific CD4+ T cell function but not CD8+ T cell function compared to patients without infection, suggesting that CD4+ T cell immunity may be a major limiting factor for the recovery of functional

HCMV immunity and protection against infection. Campath-1H also correlated with delayed CD4+ T cell HCMV function, but not CD8+ T cell function, and this may be the mechanism by which Campath-1H increases the risk of HCMV infection, as shown in Chapter One of this thesis and this has been reported by other workers (Chakrabarti et al., 2002). Further studies are indicated to characterise the recovery of HCMV specific immunity, to assess if early HCMV specific T cells are of recipient or donor origin in patients transplanted and assess cytokine responses to HCMV other than interferon gamma, such as IL2 and TNFα. The central role of HCMV specific CD4+ T helper cells may account for the beneficial effect seen with immunotherapy using polyclonal virus specific T cell clones generated by the short term culture of donor lymphocytes with monocyte derived dendritic cells pulsed with whole viral lysate (Peggs et al., 2001), resulting in large numbers of HCMV specific CD4+ T cells (Peggs et al., 2003). Adoptive therapy targeting only CD8+ HCMV specific T cells, such as those obtained by tetramer selection (Cobbold et al., 2005) may not be as effective. The use of a viral lysate derived from HCMV infected cell cultures is no longer feasible, as this practice does not confirm to good manufacturing practices.

Incorporating an assessment of HCMV specific immune function using intracellular cytokine staining provides additional clinically relevant information in stem cell transplant recipients. The monitoring of HCMV CD4+ T cell specific immune function by intracellular cytokine staining two weekly following allogeneic stem cell transplantation is labour intensive and not practical for routine practice. Restricting analysis to day

50 and day 100 post transplant may be a more practical way of identifying patients at high risk of HCMV infection, and requiring extended real time PCR monitoring for HCMV viraemia, as well as identifying potential candidates for immunotherapy.

The investigation of the CD4+ T cell immune response to HCMV viral lysate and to the HCMV proteins pp65 and IE1 showed that among stem cell transplant patients, the response to pp65 contributed less to the total response to HCMV than the total response to HCMV in normal controls. The pp65 and IE1 specific immune response of stem cell transplant patients was characterised by a broader range of epitopes when compared to normal controls. There is evidence that a broad antigenic response is important to control HCMV infection in HIV patients (Sacre et al., 2005), and this may also be the case following stem cell transplantation. This finding is also particularly relevant for HCMV specific immunotherapy, as techniques that focus on a few immunodominant epitopes, such as CD8+ tetramer selection (Cobbold et al., 2005) or use of few stimulating peptides (Rauser et al., 2004) may not be as effective as techniques that use HCMV viral lysate (Einsele et al., 2002; Peggs et al., 2001) or overlapping peptide pools of immunodominant proteins (Trivedi et al., 2005).

The study of the class II response to the HCMV proteins pp65 and IE1 in normal controls and stem cell transplant patients has identified 13 epitopes for pp65 and one epitope for IE1, including six epitopes which have not previously been described. Further work is required to generate epitope specific T cell lines, and assessing Vbeta restriction to confirm

clonality. The importance of class II molecules other then HLA DR in presenting HCMV antigens to CD4+ T cells has been demonstrated by showing that some of the identified pp65 epitopes are presented by HLA DQ molecules. This finding increases the level of complexity in predicting the likely HCMV epitope response of allogeneic transplant recipients, as this requires knowledge at a molecular level of both the alpha (for HLA DQ and DP) and beta chains. Although HCMV specific epitopes for which the class II HLA restriction is known can be used for monitoring of immune recovery following allogeneic stem cell transplantation, the complexity of class II HLA presentation presents a strong argument for the approach of using overlapping peptide pools to generate HCMV reactive T cell lines for immunotherapy irrespective of the tissue type of the donor. In the light of Sylwester's findings that proteins from at least six ORFs from HCMV are required to replicate the total response (Sylwester et al., 2005), evaluation of the response to peptide pools of immunodominant proteins other than pp65 and IE1 for stimulation of donor cells for immunotherapy is required. The optimal approach to immunotherapy that also conforms to GMP standards would be stimulation of donor derived PBMCs with overlapping peptide pools from a number of the most immunogenic HCMV proteins, and the selection of reactive CD4+ and CD8+ T cells by a technique such as the interferon gamma capture technique described by Rauser et al (Rauser et al., 2004).

This thesis has increased the knowledge of HCMV infection in allogeneic stem cell transplant recipients. This study has shown that the use of Campath-1H in vivo but not ex vivo significantly increased the risk of HCMV infection in allogeneic transplant patients. The study of real time PCR for quantitation of viral load confirmed that a higher peak viral load and faster viral replication remain risk factors for HCMV disease when pre-emptive therapy for HCMV infection is used, but the total duration of viraemia is now the most significant risk factor. Through prospective monitoring of HCMV specific immune function in stem cell transplant recipients, the central role of HCMV specific CD4+ T cells in controlling HCMV infection has been identified. A number of new HCMV specific class II epitopes with potential use in immune monitoring following transplantation have been described, and the importance of class II molecules other then HLA DB1 in presenting HCMV antigens to CD4+ T cells has been highlighted. As a result of the work in this thesis, a number of new questions have been generated which require further investigation. Clinical trials are required to identify the most appropriate threshold to initiate HCMV specific antiviral therapy and to discontinue antiviral therapy. Further prospective studies of HCMV specific immune recovery following allogeneic transplantation should be performed, including the cytokine response other than interferon gamma to HCMV, and whether early HCMV specific T cell recovery is of donor or recipient origin. Further work is required to characterise the specificity and functional status of the CD4+ T cell immune response to HCMV in allogeneic stem cell transplant recipients, including clonotypic heirachy of

responding T cells, proliferation potential in response to identified epitopes, and epitope mapping of proteins other than pp65 and IE1.

Bibliography

Reference List

Abate,D.A., Watanabe,S., and Mocarski,E.S. (2004). Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. J Virol *78*, 10995-11006.

Adler,S.P., Plotkin,S.A., Gonczol,E., Cadoz,M., Meric,C., Wang,J.B., Dellamonica,P., Best,A.M., Zahradnik,J., Pincus,S., Berencsi,K., Cox,W.I., and Gyulai,Z. (1999). A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). J. Infect. Dis. *180*, 843-846.

Ahn,K., Gruhler,A., Galocha,B., Jones,T.R., Wiertz,E.J., Ploegh,H.L., Peterson,P.A., Yang,Y., and Fruh,K. (1997). The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. Immunity. *6*, 613-621.

Akpek,G. (2002). Clinical grading in chronic graft-versus-host disease: is it time for change?
8. Leuk. Lymphoma *43*, 1211-1220.

Alp,N.J., Allport,T.D., Van,Z.J., Rodgers,B., Sissons,J.G., and Borysiewicz,L.K. (1991). Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. J. Virol. *65*, 4812-4820.

Altman,J.D., Moss,P.A., Goulder,P.J., Barouch,D.H., Heyzer-Williams,M.G., Bell,J.I., McMichael,A.J., and Davis,M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94-96.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., and Callan,M.F. (2003). Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. J. Exp. Med. *198*, 903-911.

Anders,D.G. and McCue,L.A. (1996). The human cytomegalovirus genes and proteins required for DNA synthesis. Intervirology *39*, 378-388.

Anderson,J.E., Sale,G., Appelbaum,F.R., Chauncey,T.R., and Storb,R. (1997). Allogeneic marrow transplantation for primary myelofibrosis and myelofibrosis secondary to polycythaemia vera or essential thrombocytosis. Br. J. Haematol. *98*, 1010-1016.

Appay,V., Zaunders,J.J., Papagno,L., Sutton,J., Jaramillo,A., Waters,A., Easterbrook,P., Grey,P., Smith,D., McMichael,A.J., Cooper,D.A., Rowland-Jones,S.L., and Kelleher,A.D. (2002). Characterization of CD4(+) CTLs ex vivo. J. Immunol. *168*, 5954-5958.

Arvin,A.M., Fast,P., Myers,M., Plotkin,S., and Rabinovich,R. (2004). Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. Clin Infect. Dis *39*, 233-239.

Aubert,G., Hassan-Walker,A.F., Madrigal,J.A., Emery,V.C., Morte,C., Grace,S., Koh,M.B., Potter,M., Prentice,H.G., Dodi,I.A., and Travers,P.J. (2001). Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. J. Infect. Dis. *184*, 955-963.

Aversa,F., Tabilio,A., Velardi,A., Cunningham,I., Terenzi,A., Falzetti,F., Ruggeri,L., Barbabietola,G., Aristei,C., Latini,P., Reisner,Y., and Martelli,M.F. (1998). Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N. Engl. J. Med *339*, 1186-1193.

Aversa,F., Terenzi,A., Tabilio,A., Falzetti,F., Carotti,A., Ballanti,S., Felicini,R., Falcinelli,F., Velardi,A., Ruggeri,L., Aloisi,T., Saab,J.P., Santucci,A., Perruccio,K., Martelli,M.P., Mecucci,C., Reisner,Y., and Martelli,M.F. (2005). Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. J. Clin Oncol. *23*, 3447-3454.

Avery,R., Kalaycio,M., Pohlman,B., Sobecks,R., Kuczkowski,E., Andresen,S., Mossad,S., Shamp,J., Curtis,J., Kosar,J., Sands,K., Serafin,M., and Bolwell,B. (2005). Early vancomycin-resistant enterococcus (VRE) bacteremia after allogeneic bone marrow transplantation is associated with a rapidly deteriorating clinical course
7. Bone Marrow Transplant. *35*, 497-499.

Avery,R.K., Adal,K.A., Longworth,D.L., and Bolwell,B.J. (2000). A survey of allogeneic bone marrow transplant programs in the United States regarding cytomegalovirus prophylaxis and pre-emptive therapy. Bone Marrow Transplant. *26*, 763-767.

Ayala,E., Greene,J., Sandin,R., Perkins,J., Field,T., Tate,C., Fields,K.K., and Goldstein,S. (2006). Valganciclovir is safe and effective as pre-emptive therapy for CMV infection in allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant. *37*, 851-856.

Bacigalupo,A., Brand,R., Oneto,R., Bruno,B., Socie,G., Passweg,J., Locasciulli,A., Van Lint,M.T., Tichelli,A., McCann,S., Marsh,J., Ljungman,P., Hows,J., Marin,P., and Schrezenmeier,H. (2000). Treatment of acquired severe aplastic anemia: bone marrow transplantation compared with immunosuppressive therapy--The European Group for Blood and Marrow Transplantation experience. Semin. Hematol. *37*, 69-80.

Bacigalupo,A., Locatelli,F., Lanino,E., Marsh,J., Socie,G., Maury,S., Prete,A., Locasciulli,A., Cesaro,S., and Passweg,J. (2005). Fludarabine, cyclophosphamide and anti-thymocyte globulin for alternative donor transplants in acquired severe aplastic anemia: a report from the EBMT-SAA Working Party. Bone Marrow Transplant. *36*, 947-950.

Bacigalupo,A., Tedone,E., Isaza,A., Soracco,M., Van Lint,M.T., Sanna,A., Frassoni,F., Occhini,D., Gualandi,F., Lamparelli,T., and . (1995). CMV-antigenemia after allogeneic bone marrow transplantation: correlation of CMV-antigen positive cell numbers with transplant-related mortality. Bone Marrow Transplant. *16*, 155-161.

Bader,P., Stoll,K., Huber,S., Geiselhart,A., Handgretinger,R., Niemeyer,C., Einsele,H., Schlegel,P.G., Niethammer,D., Beck,J., and Klingebiel,T. (2000). Characterization of lineage-specific chimaerism in patients with acute leukaemia and myelodysplastic syndrome after allogeneic stem cell transplantation before and after relapse. Br. J Haematol. *108*, 761-768.

Baldanti,F., Silini,E., Sarasini,A., Talarico,C.L., Stanat,S.C., Biron,K.K., Furione,M., Bono,F., Palu,G., and Gerna,G. (1995). A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. J. Virol. *69*, 796-800.

Bankier,A.T., Beck,S., Bohni,R., Brown,C.M., Cerny,R., Chee,M.S., Hutchison,C.A., III, Kouzarides,T., Martignetti,J.A., Preddie,E., and . (1991). The DNA sequence of the human cytomegalovirus genome. DNA Seq. *2*, 1-12.

Barker,J.N., Weisdorf,D.J., DeFor,T.E., Blazar,B.R., McGlave,P.B., Miller,J.S., Verfaillie,C.M., and Wagner,J.E. (2005). Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. Blood *105*, 1343-1347.

Barrett,A.J., Mavroudis,D., Tisdale,J., Molldrem,J., Clave,E., Dunbar,C., Cottler-Fox,M., Phang,S., Carter,C., Okunnieff,P., Young,N.S., and Read,E.J. (1998). T cell-depleted bone marrow transplantation and delayed T cell add-back to control acute GVHD and conserve a graft-versus-leukemia effect. Bone Marrow Transplant. *21*, 543-551.

Beatty,P.G., Clift,R.A., Mickelson,E.M., Nisperos,B.B., Flournoy,N., Martin,P.J., Sanders,J.E., Stewart,P., Buckner,C.D., Storb,R., and . (1985). Marrow transplantation from related donors other than HLA-identical siblings. N. Engl. J. Med *313*, 765-771.

Beninga,J., Kropff,B., and Mach,M. (1995). Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response. J. Gen. Virol. *76 (Pt 1)*, 153-160.

Bennett,S.R., Carbone,F.R., Karamalis,F., Flavell,R.A., Miller,J.F., and Heath,W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Bensinger,W., Singer,J., Appelbaum,F., Lilleby,K., Longin,K., Rowley,S., Clarke,E., Clift,R., Hansen,J., Shields,T., and . (1993). Autologous transplantation with peripheral blood mononuclear cells collected after administration of recombinant granulocyte stimulating factor. Blood *81*, 3158-3163.

Bensinger,W.I., Martin,P.J., Storer,B., Clift,R., Forman,S.J., Negrin,R., Kashyap,A., Flowers,M.E., Lilleby,K., Chauncey,T.R., Storb,R., and Appelbaum,F.R. (2001). Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N. Engl. J. Med *344*, 175-181.

Berencsi,K., Gyulai,Z., Gonczol,E., Pincus,S., Cox,W.I., Michelson,S., Kari,L., Meric,C., Cadoz,M., Zahradnik,J., Starr,S., and Plotkin,S. (2001). A canarypox vector-expressing cytomegalovirus (CMV) phosphoprotein 65 induces long-lasting cytotoxic T cell responses in human CMV-seronegative subjects. J. Infect. Dis. *183*, 1171-1179.

Bethge,W.A., Hegenbart,U., Stuart,M.J., Storer,B.E., Maris,M.B., Flowers,M.E., Maloney,D.G., Chauncey,T., Bruno,B., Agura,E., Forman,S.J., Blume,K.G., Niederwieser,D., Storb,R., and Sandmaier,B.M. (2004). Adoptive immunotherapy with donor lymphocyte infusions after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. Blood *103*, 790-795.

Billingham,R.E. (1966). The biology of graft-versus-host reactions. Harvey Lect. *62*, 21-78.

Billingham,R.E., Brent,L., and Medawar,P.B. (1953). Actively acquired tolerance of foreing cells. Nature *172*, 603-606.

Billstrom,M.A., Johnson,G.L., Avdi,N.J., and Worthen,G.S. (1998). Intracellular signaling by the chemokine receptor US28 during human cytomegalovirus infection. J Virol *72*, 5535-5544.

Biron,C.A., Byron,K.S., and Sullivan,J.L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells. N. Engl. J. Med *320*, 1731-1735.

Bitmansour,A.D., Waldrop,S.L., Pitcher,C.J., Khatamzas,E., Kern,F., Maino,V.C., and Picker,L.J. (2001). Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. J. Immunol. *167*, 1151-1163.

Blackburn,N.K., Besselaar,T.G., Schoub,B.D., and O'Connell,K.F. (1991). Differentiation of primary cytomegalovirus infection from reactivation using the urea denaturation test for measuring antibody avidity
8. J. Med Virol. *33*, 6-9.

Blaise,D., Kuentz,M., Fortanier,C., Bourhis,J.H., Milpied,N., Sutton,L., Jouet,J.P., Attal,M., Bordigoni,P., Cahn,J.Y., Boiron,J.M., Schuller,M.P., Moatti,J.P., and Michallet,M. (2000). Randomized trial of bone marrow versus lenograstim-primed blood cell allogeneic transplantation in patients with early-stage leukemia: a report from the Societe Francaise de Greffe de Moelle. J. Clin Oncol. *18*, 537-546.

Bodaghi,B., Jones,T.R., Zipeto,D., Vita,C., Sun,L., Laurent,L., renzana-Seisdedos,F., Virelizier,J.L., and Michelson,S. (1998). Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of

chemokines from the environment of cytomegalovirus-infected cells. J Exp Med *188*, 855-866.

Boeckh,M. and Boivin,G. (1998). Quantitation of cytomegalovirus: methodologic aspects and clinical applications. Clin Microbiol. Rev. *11*, 533-554.

Boeckh,M., Bowden,R.A., Goodrich,J.M., Pettinger,M., and Meyers,J.D. (1992). Cytomegalovirus antigen detection in peripheral blood leukocytes after allogeneic marrow transplantation. Blood *80*, 1358-1364.

Boeckh,M., Gallez-Hawkins,G.M., Myerson,D., Zaia,J.A., and Bowden,R.A. (1997). Plasma polymerase chain reaction for cytomegalovirus DNA after allogeneic marrow transplantation: comparison with polymerase chain reaction using peripheral blood leukocytes, pp65 antigenemia, and viral culture. Transplantation *64*, 108-113.

Boeckh,M., Gooley,T.A., and Bowden,R.A. (1998a). Effect of high-dose acyclovir on survival in allogeneic marrow transplant recipients who received ganciclovir at engraftment or for cytomegalovirus pp65 antigenemia. J. Infect. Dis. *178*, 1153-1157.

Boeckh,M., Gooley,T.A., Myerson,D., Cunningham,T., Schoch,G., and Bowden,R.A. (1996). Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. Blood *88*, 4063-4071.

Boeckh,M., Leisenring,W., Riddell,S.R., Bowden,R.A., Huang,M.L., Myerson,D., Stevens-Ayers,T., Flowers,M.E., Cunningham,T., and Corey,L. (2003). Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity
22. Blood *101*, 407-414.

Boeckh,M. and Nichols,W.G. (2004). The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. Blood *103*, 2003-2008.

Boeckh,M., Zaia,J.A., Jung,D., Skettino,S., Chauncey,T.R., and Bowden,R.A. (1998b). A study of the pharmacokinetics, antiviral activity, and tolerability of oral ganciclovir for CMV prophylaxis in marrow transplantation. Biol. Blood Marrow Transplant. *4*, 13-19.

Boehme,K.W., Guerrero,M., and Compton,T. (2006). Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J Immunol. *177*, 7094-7102.

Bonaros,N.E., Kocher,A., Dunkler,D., Grimm,M., Zuckermann,A., Ankersmit,J., Ehrlich,M., Wolner,E., and Laufer,G. (2004). Comparison of combined prophylaxis of cytomegalovirus hyperimmune globulin plus ganciclovir versus

Bornhauser,M., Schuler,U., Porksen,G., Naumann,R., Geissler,G., Thi
Schwerdtfeger,R., Ehninger,G., and Thiede,H.M. (1999). Mycophenol
and cyclosporine as graft-versus-host disease prophylaxis after allogen
stem cell transplantation. Transplantation *67*, 499-504.

Borysiewicz,L.K., Rodgers,B., Morris,S., Graham,S., and Sissons,J.G.
Lysis of human cytomegalovirus infected fibroblasts by natural killer c
demonstration of an interferon-independent component requiring expr
early viral proteins and characterization of effector cells. J. Immunol.
2701.

Bourgeois,C., Rocha,B., and Tanchot,C. (2002). A role for CD40 expr
CD8+ T cells in the generation of CD8+ T cell memory. Science *297*,
2063.

Bowden,R.A., Sayers,M., Flournoy,N., Newton,B., Banaji,M., Thomas
Meyers,J.D. (1986). Cytomegalovirus immune globulin and seronegati
products to prevent primary cytomegalovirus infection after marrow
transplantation. N. Engl. J. Med *314*, 1006-1010.

Bowden,R.A., Slichter,S.J., Sayers,M., Weisdorf,D., Cays,M., Schoch,
Banaji,M., Haake,R., Welk,K., Fisher,L., McCullough,J., and Miller,W
A comparison of filtered leukocyte-reduced and cytomegalovirus (CM
seronegative blood products for the prevention of transfusion-associate
infection after marrow transplant. Blood *86*, 3598-3603.

Bowden,R.A., Slichter,S.J., Sayers,M.H., Mori,M., Cays,M.J., and Me
(1991). Use of leukocyte-depleted platelets and cytomegalovirus-seron
red blood cells for prevention of primary cytomegalovirus infection aft
transplant. Blood *78*, 246-250.

Bowen,A.L., Zomas,A., Emmett,E., Matutes,E., Dyer,M.J., and Catovs
(1997). Subcutaneous CAMPATH-1H in fludarabine-resistant/relapsed
lymphocytic and B-prolymphocytic leukaemia. Br. J. Haematol. *96*, 61

Boyle,K.A. and Compton,T. (1998). Receptor-binding properties of a s
form of human cytomegalovirus glycoprotein B. J Virol *72*, 1826-183

Breems,D.A., van Putten,W.L., Huijgens,P.C., Ossenkoppele,G.J., Ver
Verdonck,L.F., Vellenga,E., De Greef,G.E., Jacky,E., Van der,L.J.,
Boogaerts,M.A., and Lowenberg,B. (2005). Prognostic index for adult
with acute myeloid leukemia in first relapse. J. Clin. Oncol. *23*, 1969-1

Britt,W.J. and Mach,M. (1996). Human cytomegalovirus glycoproteins. Intervirology *39*, 401-412.

Britt,W.J., Vugler,L., Butfiloski,E.J., and Stephens,E.B. (1990). Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. J Virol *64*, 1079-1085.

Broers,A.E., van Der,H.R., van Esser,J.W., Gratama,J.W., Henzen-Logmans,S., Kuenen-Boumeester,V., Lowenberg,B., and Cornelissen,J.J. (2000). Increased transplant-related morbidity and mortality in CMV-seropositive patients despite highly effective prevention of CMV disease after allogeneic T-cell-depleted stem cell transplantation. Blood *95*, 2240-2245.

Bronke,C., Palmer,N.M., Westerlaken,G.H., Toebes,M., van Schijndel,G.M., Purwaha,V., van Meijgaarden,K.E., Schumacher,T.N., van,B.D., Tesselaar,K., and Geluk,A. (2005). Direct ex vivo detection of HLA-DR3-restricted cytomegalovirus- and Mycobacterium tuberculosis-specific CD4+ T cells. Hum. Immunol. *66*, 950-961.

Browne,E.P. and Shenk,T. (2003). Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. Proc. Natl. Acad. Sci. U. S. A *100*, 11439-11444.

Brunstein,C.G., Weisdorf,D.J., DeFor,T., Barker,J.N., Tolar,J., van Burik,J.A., and Wagner,J.E. (2006). Marked increased risk of Epstein-Barr virus-related complications with the addition of antithymocyte globulin to a nonmyeloablative conditioning prior to unrelated umbilical cord blood transplantation 1. Blood *108*, 2874-2880.

Bunjes,D. (2000). T cell depletion of allogeneic stem cell grafts with anti-CD 52 monoclonal antibodies: the Ulm experience from 1983-1999. Transfus. Sci. *23*, 151-162.

Burlingham,W.J., Grailer,A.P., Heisey,D.M., Claas,F.H., Norman,D., Mohanakumar,T., Brennan,D.C., de,F.H., van,G.T., Pirsch,J.D., Sollinger,H.W., and Bean,M.A. (1998). The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. N. Engl. J. Med *339*, 1657-1664.

Burnett,A.K., Wheatley,K., Goldstone,A.H., Stevens,R.F., Hann,I.M., Rees,J.H., and Harrison,G. (2002). The value of allogeneic bone marrow transplant in patients with acute myeloid leukaemia at differing risk of relapse: results of the UK MRC AML 10 trial. Br. J. Haematol. *118*, 385-400.

Burrows,S.R.,Elkington,R.A.,Miles,J.J.,Green,K.J.,Walker,S.,Haryana,S.M.,Mos s,D.J.,Dunckley,H.,Burrows,J.M.,Khana,R. (2003). Promiscous CTL recognition of viral epitopes on multiple humen leukocyte antigens:biological validation of the proposed HLA A24 supertype. J Immunol *171*, 1407-1412.

Cardone,G., Winkler,D.C., Trus,B.L., Cheng,N., Heuser,J.E., Newcomb,W.W., Brown,J.C., and Steven,A.C. (2006). Visualization of the herpes simplex virus portal in situ by cryo-electron tomography. Virology.

Carreiras,M., Montagnani,S., and Layrisse,Z. (2002). Preeclampsia: a multifactorial disease resulting from the interaction of the feto-maternal HLA genotype and HCMV infection. Am J Reprod. Immunol. *48*, 176-183.

Castillo,J.P., Frame,F.M., Rogoff,H.A., Pickering,M.T., Yurochko,A.D., and Kowalik,T.F. (2005). Human cytomegalovirus IE1-72 activates ataxia telangiectasia mutated kinase and a p53/p21-mediated growth arrest response. J Virol *79*, 11467-11475.

Cavins,J.A., Kasakura,S., Thomas,E.D., and Ferrebee,J.W. (1962). Recovery of lethally irradiated dogs following infusion of autologous marrow stored at low temperature in dimethylsulphoxide. Blood *20*, 730-734.

Cerrina,J., Le Roy,L.F., Herve,P.H., Parquin,F., Harari,S., Chapelier,A., Simoneau,G., Vouhe,P., and Dartevelle,P.H. (1992). Role of CMV pneumonia in the development of obliterative bronchiolitis in heart-lung and double-lung transplant recipients
16. Transpl. Int. *5 Suppl 1*, S242-S245.

Cesaro,S., Murrone,A., Mengoli,C., Pillon,M., Biasolo,M.A., Calore,E., Tridello,G., Varotto,S., Alaggio,R., Zanesco,L., Palu,G., and Messina,C. (2005). The real-time polymerase chain reaction-guided modulation of immunosuppression enables the pre-emptive management of Epstein-Barr virus reactivation after allogeneic haematopoietic stem cell transplantation
1. Br. J. Haematol. *128*, 224-233.

Cha,T.A., Tom,E., Kemble,G.W., Duke,G.M., Mocarski,E.S., and Spaete,R.R. (1996). Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J. Virol. *70*, 78-83.

Chakrabarti,S., MacDonald,D., Hale,G., Holder,K., Turner,V., Czarnecka,H., Thompson,J., Fegan,C., Waldmann,H., and Milligan,D.W. (2003). T-cell depletion with Campath-1H "in the bag" for matched related allogeneic peripheral blood stem cell transplantation is associated with reduced graft-versus-host disease, rapid immune constitution and improved survival. Br. J Haematol. *121*, 109-118.

Chakrabarti,S., Mackinnon,S., Chopra,R., Kottaridis,P.D., Peggs,K., O'Gorman,P., Chakraverty,R., Marshall,T., Osman,H., Mahendra,P., Craddock,C., Waldmann,H., Hale,G., Fegan,C.D., Yong,K., Goldstone,A.H., Linch,D.C., and Milligan,D.W. (2002a). High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. Blood *99*, 4357-4363.

Chakrabarti,S., Mautner,V., Osman,H., Collingham,K.E., Fegan,C.D., Klapper,P.E., Moss,P.A., and Milligan,D.W. (2002b). Adenovirus infections following allogeneic stem cell transplantation: incidence and outcome in relation

to graft manipulation, immunosuppression, and immune recovery
1. Blood *100*, 1619-1627.

Chakraverty,R., Dimopoulou,M., Glanville,J., Thomson,K., Peggs,K., Milligan,D., Cook,G., Jackson,G., Parker,A., Hunter,A., Russell,N., Pearce,R., and Mackinnon,S. (2006). De-Escalation of the Alemtuzumab Dose Prior to Nonmyeloablative HLA-Identical Sibling Transplantation: Crucial Role of Timing. ASH Annual Meeting Abstracts *108*, 2901.

Chalupny,N.J., Rein-Weston,A., Dosch,S., and Cosman,D. (2006). Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. Biochem. Biophys. Res. Commun. *346*, 175-181.

Chandler,S.H. and McDougall,J.K. (1986). Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus. J. Gen. Virol. *67 (Pt 10)*, 2179-2192.

Chang,W.L., Baumgarth,N., Yu,D., and Barry,P.A. (2004). Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. J Virol *78*, 8720-8731.

Chapman,T.L., Heikeman,A.P., and Bjorkman,P.J. (1999). The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. Immunity. *11*, 603-613.

Chee,M.S., Bankier,A.T., Beck,S., Bohni,R., Brown,C.M., Cerny,R., Horsnell,T., Hutchison,C.A., III, Kouzarides,T., Martignetti,J.A., and . (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. *154*, 125-169.

Chen,D.H., Jiang,H., Lee,M., Liu,F., and Zhou,Z.H. (1999). Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. Virology *260*, 10-16.

Chklovskaia,E., Nowbakht,P., Nissen,C., Gratwohl,A., Bargetzi,M., and Wodnar-Filipowicz,A. (2004). Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: effects of endogenous flt3 ligand
21. Blood *103*, 3860-3868.

Chopra,R., Goldstone,A.H., Pearce,R., Philip,T., Petersen,F., Appelbaum,F., De,V.E., and Ernst,P. (1992). Autologous versus allogeneic bone marrow transplantation for non-Hodgkin's lymphoma: a case-controlled analysis of the European Bone Marrow Transplant Group Registry data. J. Clin. Oncol. *10*, 1690-1695.

Chou,S., Erice,A., Jordan,M.C., Vercellotti,G.M., Michels,K.R., Talarico,C.L., Stanat,S.C., and Biron,K.K. (1995a). Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. *171*, 576-583.

Chou,S., Guentzel,S., Michels,K.R., Miner,R.C., and Drew,W.L. (1995b). Frequency of UL97 phosphotransferase mutations related to ganciclovir resistance in clinical cytomegalovirus isolates. J. Infect. Dis. *172*, 239-242.

Chou,S.W. and Dennison,K.M. (1991). Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization-related epitopes
2. J. Infect. Dis. *163*, 1229-1234.

Clift,R.A., Appelbaum,F.R., and Thomas,E.D. (1993). Treatment of chronic myeloid leukemia by marrow transplantation. Blood *82*, 1954-1956.

Cobbold,M., Khan,N., Pourgheysari,B., Tauro,S., McDonald,D., Osman,H., Assenmacher,M., Billingham,L., Steward,C., Crawley,C., Olavarria,E., Goldman,J., Chakraverty,R., Mahendra,P., Craddock,C., and Moss,P.A. (2005). Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers
2. J. Exp Med *202*, 379-386.

Colberg-Poley,A.M. (1996). Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36-38, UL115-119, TRS1/IRS1 and US3 loci. Intervirology *39*, 350-360.

Cole,R. and Kuttner,A.G. (1926). Filterable virus present in the salivary glands of guinea pigs. J Exp Medicine *44*, 855-873.

Collins,R.H., Jr., Shpilberg,O., Drobyski,W.R., Porter,D.L., Giralt,S., Champlin,R., Goodman,S.A., Wolff,S.N., Hu,W., Verfaillie,C., List,A., Dalton,W., Ognoskie,N., Chetrit,A., Antin,J.H., and Nemunaitis,J. (1997). Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J. Clin. Oncol. *15*, 433-444.

Compton,T., Kurt-Jones,E.A., Boehme,K.W., Belko,J., Latz,E., Golenbock,D.T., and Finberg,R.W. (2003). Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J Virol *77*, 4588-4596.

Cook,M., Briggs,D., Craddock,C., Mahendra,P., Milligan,D., Fegan,C., Darbyshire,P., Lawson,S., Boxall,E., and Moss,P. (2006). Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell replete stem cell transplantation. Blood *107*, 1230-1232.

Cope,A.V., Sabin,C., Burroughs,A., Rolles,K., Griffiths,P.D., and Emery,V.C. (1997). Interrelationships among quantity of human cytomegalovirus (HCMV) DNA in blood, donor-recipient serostatus, and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation. J Infect. Dis *176*, 1484-1490.

Cornelissen,J.J., van Putten,W.L., Verdonck,L.F., Theobald,M., Jacky,E., Daenen,S.M., van Marwijk,K.M., Wijermans,P., Schouten,H., Huijgens,P.C., van der,L.H., Fey,M., Ferrant,A., Maertens,J., Gratwohl,A., and Lowenberg,B. (2007). Results of a HOVON/SAKK donor versus no-donor analysis of

myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom? Blood *109*, 3658-3666.

Couriel,D., Canosa,J., Engler,H., Collins,A., Dunbar,C., and Barrett,A.J. (1996). Early reactivation of cytomegalovirus and high risk of interstitial pneumonitis following T-depleted BMT for adults with hematological malignancies. Bone Marrow Transplant. *18*, 347-353.

Cox,D.R. (1972). Regression models and life tables (with discussion). J R Stat B *34*, 187-220.

Craddock,C., Szydlo,R.M., Dazzi,F., Olavarria,E., Cwynarski,K., Yong,A., Brookes,P., de la,F.J., Kanfer,E., Apperley,J.F., and Goldman,J.M. (2001). Cytomegalovirus seropositivity adversely influences outcome after T-depleted unrelated donor transplant in patients with chronic myeloid leukaemia: the case for tailored graft-versus-host disease prophylaxis. Br. J. Haematol. *112*, 228-236.

Craddock,C., Szydlo,R.M., Klein,J.P., Dazzi,F., Olavarria,E., van,R.F., Pocock,C., Cwynarski,K., Apperley,J.F., and Goldman,J.M. (2000). Estimating leukemia-free survival after allografting for chronic myeloid leukemia: a new method that takes into account patients who relapse and are restored to complete remission. Blood *96*, 86-90.

Craig,J.M., Macauley,J.C., Weller,T.H., and Wirth,P. (1957). Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. Proc. Soc. Exp Biol Med *94*, 4-12.

Crawley,C., Szydlo,R., Lalancette,M., Bacigalupo,A., Lange,A., Brune,M., Juliusson,G., Nagler,A., Gratwohl,A., Passweg,J., Komarnicki,M., Vitek,A., Mayer,J., Zander,A., Sierra,J., Rambaldi,A., Ringden,O., Niederwieser,D., and Apperley,J.F. (2005). Outcomes of reduced-intensity transplantation for chronic myeloid leukemia: an analysis of prognostic factors from the Chronic Leukemia Working Party of the EBMT. Blood *106*, 2969-2976.

Crippa,F., Corey,L., Chuang,E.L., Sale,G., and Boeckh,M. (2001). Virological, clinical, and ophthalmologic features of cytomegalovirus retinitis after hematopoietic stem cell transplantation
9. Clin Infect. Dis. *32*, 214-219.

Crump,C.M., Hung,C.H., Thomas,L., Wan,L., and Thomas,G. (2003). Role of PACS-1 in trafficking of human cytomegalovirus glycoprotein B and virus production. J Virol *77*, 11105-11113.

Cui,J., Han,L.Y., Lin,H.H., Zhang,H.L., Tang,Z.Q., Zheng,C.J., Cao,Z.W., and Chen,Y.Z. (2007). Prediction of MHC-binding peptides of flexible lengths from sequence-derived structural and physicochemical properties. Mol. Immunol. *44*, 866-877.

Cundy,K.C., Li,Z.H., Hitchcock,M.J., and Lee,W.A. (1996). Pharmacokinetics of cidofovir in monkeys. Evidence for a prolonged elimination phase representing

phosphorylated drug
46. Drug Metab Dispos. *24*, 738-744.

Cwynarski,K., Ainsworth,J., Cobbold,M., Wagner,S., Mahendra,P., Apperley,J., Goldman,J., Craddock,C., and Moss,P.A. (2001). Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood *97*, 1232-1240.

Daly,A., Song,K., Nevill,T., Nantel,S., Toze,C., Hogge,D., Forrest,D., Lavoie,J., Sutherland,H., Shepherd,J., Hasegawa,W., Lipton,J., Messner,H., and Kiss,T. (2003). Stem cell transplantation for myelofibrosis: a report from two Canadian centers. Bone Marrow Transplant. *32*, 35-40.

Dausset,J. (1958). [Iso-leuko-antibodies.]. Acta Hematol. *20*, 156-166.

Davignon,J.L., Castanie,P., Yorke,J.A., Gautier,N., Clement,D., and Davrinche,C. (1996). Anti-human cytomegalovirus activity of cytokines produced by CD4+ T-cell clones specifically activated by IE1 peptides in vitro. J. Virol. *70*, 2162-2169.

Day,C.L., Seth,N.P., Lucas,M., Appel,H., Gauthier,L., Lauer,G.M., Robbins,G.K., Szczepiorkowski,Z.M., Casson,D.R., Chung,R.T., Bell,S., Harcourt,G., Walker,B.D., Klenerman,P., and Wucherpfennig,K.W. (2003). Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. J. Clin. Invest *112*, 831-842.

Dazzi,F., Szydlo,R.M., Cross,N.C., Craddock,C., Kaeda,J., Kanfer,E., Cwynarski,K., Olavarria,E., Yong,A., Apperley,J.F., and Goldman,J.M. (2000). Durability of responses following donor lymphocyte infusions for patients who relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. Blood *96*, 2712-2716.

de Clercq E. (2003). New inhibitors of human cytomegalovirus (HCMV) on the horizon
2. J. Antimicrob. Chemother. *51*, 1079-1083.

de Lima M., van Besien,K.W., Giralt,S.A., Khouri,I.F., Mehra,R., Andersson,B.S., Przepiorka,D., Gajewski,J.L., Korbling,M., and Champlin,R.E. (1997). Bone marrow transplantation after failure of autologous transplant for non-Hodgkin's lymphoma. Bone Marrow Transplant. *19*, 121-127.

de Witte,T., Hoogenhout,J., de,P.B., Holdrinet,R., Janssen,J., Wessels,J., van,D.W., Hustinx,T., and Haanen,C. (1986). Depletion of donor lymphocytes by counterflow centrifugation successfully prevents acute graft-versus-host disease in matched allogeneic marrow transplantation. Blood *67*, 1302-1308.

de Witte,T., Schattenberg,A., Van Dijk,B.A., Galama,J., Olthuis,H., Van der Meer,J.W., and Kunst,V.A. (1990). Prevention of primary cytomegalovirus infection after allogeneic bone marrow transplantation by using leukocyte-poor random blood products from cytomegalovirus-unscreened blood-bank donors. Transplantation *50*, 964-968.

Deayton,J.R., Sabin,C.A., Johnson,M.A., Emery,V.C., Wilson,P., and Griffiths,P.D. (2004). Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. Lancet *363*, 2116-2121.

Deayton,J.R., Wilson,P., Sabin,C.A., Davey,C.C., Johnson,M.A., Emery,V.C., and Griffiths,P.D. (2000). Changes in the natural history of cytomegalovirus retinitis following the introduction of highly active antiretroviral therapy 20. AIDS *14*, 1163-1170.

Deeg,H.J., Gooley,T.A., Flowers,M.E., Sale,G.E., Slattery,J.T., Anasetti,C., Chauncey,T.R., Doney,K., Georges,G.E., Kiem,H.P., Martin,P.J., Petersdorf,E.W., Radich,J., Sanders,J.E., Sandmaier,B.M., Warren,E.H., Witherspoon,R.P., Storb,R., and Appelbaum,F.R. (2003). Allogeneic hematopoietic stem cell transplantation for myelofibrosis. Blood *102*, 3912-3918.

Deeg,H.J., O'Donnell,M., Tolar,J., Agarwal,R., Harris,R.E., Feig,S.A., Territo,M.C., Collins,R.H., McSweeney,P.A., Copelan,E.A., Khan,S.P., Woolfrey,A., and Storer,B. (2006). Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. Blood *108*, 1485-1491.

Deeg,H.J., Sullivan,K.M., Buckner,C.D., Storb,R., Appelbaum,F.R., Clift,R.A., Doney,K., Sanders,J.E., Witherspoon,R.P., and THOMAS,E.D. (1986). Marrow transplantation for acute nonlymphoblastic leukemia in first remission: toxicity and long-term follow-up of patients conditioned with single dose or fractionated total body irradiation. Bone Marrow Transplant. *1*, 151-157.

Delgado,J., Thomson,K., Russell,N., Ewing,J., Stewart,W., Cook,G., Devereux,S., Lovell,R., Chopra,R., Marks,D.I., Mackinnon,S., and Milligan,D.W. (2006). Results of alemtuzumab-based reduced-intensity allogeneic transplantation for chronic lymphocytic leukemia: a British Society of Blood and Marrow Transplantation Study. Blood *107*, 1724-1730.

Deray,G., Martinez,F., Katlama,C., Levaltier,B., Beaufils,H., Danis,M., Rozenheim,M., Baumelou,A., Dohin,E., Gentilini,M., and . (1989). Foscarnet nephrotoxicity: mechanism, incidence and prevention. Am. J. Nephrol. *9*, 316-321.

Devergie,A., Apperley,J.F., Labopin,M., Madrigal,A., Jacobsen,N., Carreras,E., Prentice,H.G., Jouet,J.P., Kolb,H.J., Herstenstein,B., Bacigalupo,A., Evensen,S.A., Ljungman,P., De,W.T., Reiffers,J., Nagler,A., Clark,R.E., Goldman,J.M., and Gratwohl,A. (1997). European results of matched unrelated donor bone marrow transplantation for chronic myeloid leukemia. Impact of HLA class II matching. Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant. *20*, 11-19.

Devine,S.M., Hoffman,R., Verma,A., Shah,R., Bradlow,B.A., Stock,W., Maynard,V., Jessop,E., Peace,D., Huml,M., Thomason,D., Chen,Y.H., and van,B.K. (2002). Allogeneic blood cell transplantation following reduced-

intensity conditioning is effective therapy for older patients with myelofibrosis with myeloid metaplasia. Blood *99*, 2255-2258.

Diamond,D.J., York,J., Sun,J.Y., Wright,C.L., and Forman,S.J. (1997). Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood *90*, 1751-1767.

Digel,M., Sampaio,K.L., Jahn,G., and Sinzger,C. (2006). Evidence for direct transfer of cytoplasmic material from infected to uninfected cells during cell-associated spread of human cytomegalovirus. J Clin Virol *37*, 10-20.

Dittmer,A., Drach,J.C., Townsend,L.B., Fischer,A., and Bogner,E. (2005). Interaction of the putative human cytomegalovirus portal protein pUL104 with the large terminase subunit pUL56 and its inhibition by benzimidazole-D-ribonucleosides. J Virol *79*, 14660-14667.

Dokal,I. (2003). Inherited aplastic anaemia
18. Hematol. J. *4*, 3-9.

Dolan,A., Cunningham,C., Hector,R.D., Hassan-Walker,A.F., Lee,L., Addison,C., Dargan,D.J., McGeoch,D.J., Gatherer,D., Emery,V.C., Griffiths,P.D., Sinzger,C., McSharry,B.P., Wilkinson,G.W., and Davison,A.J. (2004). Genetic content of wild-type human cytomegalovirus. J. Gen. Virol. *85*, 1301-1312.

Dolstra,H., Van de Wiel-van Kemenade, De,W.T., and Preijers,F. (1996). Clonal predominance of cytomegalovirus-specific CD8+ cytotoxic T lymphocytes in bone marrow recipients. Bone Marrow Transplant. *18*, 339-345.

Doney,K.C., Chauncey,T., and Appelbaum,F.R. (2002). Allogeneic related donor hematopoietic stem cell transplantation for treatment of chronic lymphocytic leukemia. Bone Marrow Transplant. *29*, 817-823.

Donnes,P. and Kohlbacher,O. (2005). Integrated modeling of the major events in the MHC class I antigen processing pathway. Protein Sci. *14*, 2132-2140.

Doytchinova,I.A. and Flower,D.R. (2006). Class I T-cell epitope prediction: improvements using a combination of proteasome cleavage, TAP affinity, and MHC binding. Mol. Immunol. *43*, 2037-2044.

Dreger,P., Brand,R., Milligan,D., Corradini,P., Finke,J., Lambertenghi,D.G., Martino,R., Russell,N., van,B.A., Michallet,M., and Niederwieser,D. (2005). Reduced-intensity conditioning lowers treatment-related mortality of allogeneic stem cell transplantation for chronic lymphocytic leukemia: a population-matched analysis. Leukemia *19*, 1029-1033.

Dreger,P., Suttorp,M., Haferlach,T., Loffler,H., Schmitz,N., and Schroyens,W. (1993). Allogeneic granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells for treatment of engraftment failure after bone marrow transplantation. Blood *81*, 1404-1407.

Dreger,P., Viehmann,K., Steinmann,J., Eckstein,V., Muller-Ruchholtz,W., Loffler,H., and Schmitz,N. (1995). G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: comparison of T cell depletion strategies using different CD34+ selection systems or CAMPATH-1. Exp. Hematol. *23*, 147-154.

Einsele,H., Ehninger,G., Hebart,H., Wittkowski,K.M., Schuler,U., Jahn,G., Mackes,P., Herter,M., Klingebiel,T., Loffler,J., Wagner,S., and Muller,C.A. (1995). Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood *86*, 2815-2820.

Einsele,H., Ehninger,G., Steidle,M., Vallbracht,A., Muller,M., Schmidt,H., Saal,J.G., Waller,H.D., and Muller,C.A. (1991). Polymerase chain reaction to evaluate antiviral therapy for cytomegalovirus disease
1. Lancet *338*, 1170-1172.

Einsele,H., Hebart,H., Kauffmann-Schneider,C., Sinzger,C., Jahn,G., Bader,P., Klingebiel,T., Dietz,K., Loffler,J., Bokemeyer,C., Muller,C.A., and Kanz,L. (2000). Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. Bone Marrow Transplant. *25*, 757-763.

Einsele,H., Reusser,P., Bornhauser,M., Kalhs,P., Ehninger,G., Hebart,H., Chalandon,Y., Kroger,N., Hertenstein,B., and Rohde,F. (2006). Oral valganciclovir leads to higher exposure to ganciclovir than intravenous ganciclovir in patients following allogeneic stem cell transplantation. Blood *107*, 3002-3008.

Einsele,H., Roosnek,E., Rufer,N., Sinzger,C., Riegler,S., Loffler,J., Grigoleit,U., Moris,A., Rammensee,H.G., Kanz,L., Kleihauer,A., Frank,F., Jahn,G., and Hebart,H. (2002). Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy
3. Blood *99*, 3916-3922.

Elek,S.D. and Stern,H. (1974). Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. Lancet *1*, 1-5.

Elkington,R., Shoukry,N.H., Walker,S., Crough,T., Fazou,C., Kaur,A., Walker,C.M., and Khanna,R. (2004). Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H. Eur. J. Immunol. *34*, 3216-3226.

Emanuel,D., Cunningham,I., Jules-Elysee,K., Brochstein,J.A., Kernan,N.A., Laver,J., Stover,D., White,D.A., Fels,A., Polsky,B., and . (1988). Cytomegalovirus pneumonia after bone marrow transplantation successfully treated with the combination of ganciclovir and high-dose intravenous immune globulin. Ann Intern Med *109*, 777-782.

Emery,V.C. (1998). Cytomegalovirus drug resistance
30. Antivir. Ther. *3*, 239-242.

Emery,V.C. (2000). Tuning to the right frequency: cytotoxic T lymphocytes and cytomegalovirus. Transplantation *69*, 2241-2242.

Emery,V.C., Cope,A.V., Bowen,E.F., Gor,D., and Griffiths,P.D. (1999). The dynamics of human cytomegalovirus replication in vivo. J. Exp. Med *190*, 177-182.

Emery,V.C., Hassan-Walker,A.F., Burroughs,A.K., and Griffiths,P.D. (2002). Human cytomegalovirus (HCMV) replication dynamics in HCMV-naive and -experienced immunocompromised hosts. J Infect. Dis *185*, 1723-1728.

Emery,V.C., Sabin,C.A., Cope,A.V., Gor,D., Hassan-Walker,A.F., and Griffiths,P.D. (2000). Application of viral-load kinetics to identify patients who develop cytomegalovirus disease after transplantation. Lancet *355*, 2032-2036.

Engelhard,D., Weinberg,M., Or,R., Shaked,O., Naparstek,E., Haikin,H., Slavin,S., and Sarov,I. (1991). Immunoglobulins A, G, and M to cytomegalovirus during recurrent infection in recipients of allogeneic bone marrow transplantation. J. Infect. Dis. *163*, 628-630.

Engstrand,M., Tournay,C., Peyrat,M.A., Eriksson,B.M., Wadstrom,J., Wirgart,B.Z., Romagne,F., Bonneville,M., Totterman,T.H., and Korsgren,O. (2000). Characterization of CMVpp65-specific CD8+ T lymphocytes using MHC tetramers in kidney transplant patients and healthy participants. Transplantation *69*, 2243-2250.

Enright,H., Haake,R., Weisdorf,D., Ramsay,N., McGlave,P., Kersey,J., Thomas,W., McKenzie,D., and Miller,W. (1993). Cytomegalovirus pneumonia after bone marrow transplantation. Risk factors and response to therapy. Transplantation *55*, 1339-1346.

Erard,V., Kim,H.W., Corey,L., Limaye,A., Huang,M.L., Myerson,D., Davis,C., and Boeckh,M. (2005). BK DNA viral load in plasma: evidence for an association with hemorrhagic cystitis in allogeneic hematopoietic cell transplant recipients
21. Blood *106*, 1130-1132.

Erice,A., Borrell,N., Li,W., Miller,W.J., and Balfour,H.H., Jr. (1998). Ganciclovir susceptibilities and analysis of UL97 region in cytomegalovirus (CMV) isolates from bone marrow recipients with CMV disease after antiviral prophylaxis. J. Infect. Dis. *178*, 531-534.

Erice,A., Holm,M.A., Sanjuan,M.V., Dunn,D.L., Gill,P.C., and Balfour,H.H., Jr. (1995). Evaluation of CMV-vue antigenemia assay for rapid detection of cytomegalovirus in mixed-leukocyte blood fractions. J. Clin Microbiol. *33*, 1014-1015.

Escalon,M.P., Champlin,R.E., Saliba,R.M., Acholonu,S.A., Hosing,C., Fayad,L., Giralt,S., Ueno,N.T., Maadani,F., Pro,B., Donato,M., McLaughlin,P., and Khouri,I.F. (2004). Nonmyeloablative allogeneic hematopoietic transplantation: a promising salvage therapy for patients with non-Hodgkin's lymphoma whose

disease has failed a prior autologous transplantation. J. Clin. Oncol. *22*, 2419-2423.

Ettinger,R.A. and Kwok,W.W. (1998). A peptide binding motif for HLA-DQA1*0102/DQB1*0602, the class II MHC molecule associated with dominant protection in insulin-dependent diabetes mellitus. J. Immunol. *160*, 2365-2373.

Fallen,P.R., McGreavey,L., Madrigal,J.A., Potter,M., Ethell,M., Prentice,H.G., Guimaraes,A., and Travers,P.J. (2003). Factors affecting reconstitution of the T cell compartment in allogeneic haematopoietic cell transplant recipients
8. Bone Marrow Transplant. *32*, 1001-1014.

Fateh-Moghadam,S., Bocksch,W., Wessely,R., Jager,G., Hetzer,R., and Gawaz,M. (2003). Cytomegalovirus infection status predicts progression of heart-transplant vasculopathy
1. Transplantation *76*, 1470-1474.

Faulkner,R.D., Craddock,C., Byrne,J.L., Mahendra,P., Haynes,A.P., Prentice,H.G., Potter,M., Pagliuca,A., Ho,A., Devereux,S., McQuaker,G., Mufti,G., Yin,J.L., and Russell,N.H. (2004). BEAM-alemtuzumab reduced-intensity allogeneic stem cell transplantation for lymphoproliferative diseases: GVHD, toxicity, and survival in 65 patients. Blood *103*, 428-434.

Fefer,A., Cheever,M.A., Thomas,E.D., Boyd,C., Ramberg,R., Glucksberg,H., Buckner,C.D., and Storb,R. (1979). Disappearance of Ph1-positive cells in four patients with chronic granulocytic leukemia after chemotherapy, irradiation and marrow transplantation from an identical twin. N. Engl. J. Med. *300*, 333-337.

Feire,A.L., Koss,H., and Compton,T. (2004). Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc. Natl. Acad. Sci. U. S. A *101*, 15470-15475.

Ferlin,W., Glaichenhaus,N., and Mougneau,E. (2000). Present difficulties and future promise of MHC multimers in autoimmune exploration. Curr. Opin. Immunol. *12*, 670-675.

Feuchtinger,T., Lang,P., and Handgretinger,R. (2007). Adenovirus infection after allogeneic stem cell transplantation
5. Leuk. Lymphoma *48*, 244-255.

Feuchtinger,T., Matthes-Martin,S., Richard,C., Lion,T., Fuhrer,M., Hamprecht,K., Handgretinger,R., Peters,C., Schuster,F.R., Beck,R., Schumm,M., Lotfi,R., Jahn,G., and Lang,P. (2006). Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation
19. Br. J. Haematol. *134*, 64-76.

Fiddian,P., Sabin,C.A., and Griffiths,P.D. (2002). Valacyclovir provides optimum acyclovir exposure for prevention of cytomegalovirus and related outcomes after organ transplantation. J. Infect. Dis. *186 Suppl 1*, S110-S115.

Fish,K.N., Britt,W., and Nelson,J.A. (1996). A novel mechanism for persistence of human cytomegalovirus in macrophages. J Virol *70*, 1855-1862.

Fleckenstein,B., Jung,G., and Wiesmuller,K.H. (1999). Quantitative analysis of peptide-MHC class II interaction. Semin. Immunol. *11*, 405-416.

Forman,S.J. and Zaia,J.A. (1994). Treatment and prevention of cytomegalovirus pneumonia after bone marrow transplantation: where do we stand? Blood *83*, 2392-2398.

Fowler,K.B., McCollister,F.P., Dahle,A.J., Boppana,S., Britt,W.J., and Pass,R.F. (1997). Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection. J Pediatr. *130*, 624-630.

Fowler,K.B., Stagno,S., Pass,R.F., Britt,W.J., Boll,T.J., and Alford,C.A. (1992). The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. N. Engl. J Med *326*, 663-667.

Fox,J.C., Griffiths,P.D., and Emery,V.C. (1992). Quantification of human cytomegalovirus DNA using the polymerase chain reaction. J. Gen. Virol. *73 (Pt 9)*, 2405-2408.

Francisci,D., Aversa,F., Coricelli,V., Carotti,A., Canovari,B., Falcinelli,F., Belfiori,B., Aloisi,T., Baldelli,F., Martelli,M.F., and Stagni,G. (2006). Prevalence, incidence and clinical outcome of hepatitis B virus and hepatitis C virus hepatitis in patients undergoing allogeneic hematopoietic stem cell transplantation between 2001 and 2004
9. Haematologica *91*, 980-982.

Frickhofen,N., Heimpel,H., Kaltwasser,J.P., and Schrezenmeier,H. (2003). Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. Blood *101*, 1236-1242.

Friede,T., Gnau,V., Jung,G., Keilholz,W., Stevanovic,S., and Rammensee,H.G. (1996). Natural ligand motifs of closely related HLA-DR4 molecules predict features of rheumatoid arthritis associated peptides
4. Biochim. Biophys. Acta *1316*, 85-101.

Fries,B.C., Riddell,S.R., Kim,H.W., Corey,L., Dahlgren,C., Woolfrey,A., and Boeckh,M. (2005). Cytomegalovirus disease before hematopoietic cell transplantation as a risk for complications after transplantation. Biol. Blood Marrow Transplant. *11*, 136-148.

Gallez-Hawkins,G., Li,X., Franck,A.E., Thao,L., Lacey,S.F., Diamond,D.J., and Zaia,J.A. (2004). DNA and low titer, helper-free, recombinant AAV prime-boost vaccination for cytomegalovirus induces an immune response to CMV-pp65 and CMV-IE1 in transgenic HLA A*0201 mice
16. Vaccine *23*, 819-826.

Gallot,G., Vivien,R., Ibisch,C., Lule,J., Davrinche,C., Gaschet,J., and Vie,H. (2001). Purification of Ag-specific T lymphocytes after direct peripheral blood

mononuclear cell stimulation followed by CD25 selection. I. Application to CD4(+) or CD8(+) cytomegalovirus phosphoprotein pp65 epitope determination. J. Immunol. *167*, 4196-4206.

Gamadia,L.E., Remmerswaal,E.B., Weel,J.F., Bemelman,F., van Lier,R.A., and ten,B., I (2003). Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. Blood *101*, 2686-2692.

Gamadia,L.E., Rentenaar,R.J., Baars,P.A., Remmerswaal,E.B., Surachno,S., Weel,J.F., Toebes,M., Schumacher,T.N., ten,B., I, and van Lier,R.A. (2001). Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers
2. Blood *98*, 754-761.

Garcia-Gallo,C.L., Gil,P.U., Laporta,R., Carreno,M.C., de,P.A., and Ferreiro,M.J. (2005). Is gammaglobulin anti-CMV warranted in lung transplantation? Transplant. Proc. *37*, 4043-4045.

Gatti,R.A., Meuwissen,H.J., Allen,H.D., Hong,R., and Good,R.A. (1968). Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet *2*, 1366-1369.

Gautier,N., Chavant,E., Prieur,E., Monsarrat,B., Mazarguil,H., Davrinche,C., Gairin,J.E., and Davignon,J.L. (1996). Characterization of an epitope of the human cytomegalovirus protein IE1 recognized by a CD4+ T cell clone. Eur. J. Immunol. *26*, 1110-1117.

Gebert,S., Schmolke,S., Sorg,G., Floss,S., Plachter,B., and Stamminger,T. (1997). The UL84 protein of human cytomegalovirus acts as a transdominant inhibitor of immediate-early-mediated transactivation that is able to prevent viral replication. J Virol *71*, 7048-7060.

Gehrz,R.C., Fuad,S., Liu,Y.N., and Bach,F.H. (1987). HLA class II restriction of T helper cell response to cytomegalovirus (CMV). I. Immunogenetic control of restriction. J. Immunol. *138*, 3145-3151.

Gehrz,R.C., Linner,K.M., Christianson,W.R., Ohm,A.E., and Balfour,H.H., Jr. (1982). Cytomegalovirus infection in infancy: virological and immunological studies. Clin Exp Immunol. *47*, 27-33.

Gerna,G., Furione,M., Baldanti,F., Percivalle,E., Comoli,P., and Locatelli,F. (1995). Quantitation of human cytomegalovirus DNA in bone marrow transplant recipients. Br. J. Haematol. *91*, 674-683.

Gerna,G., Furione,M., Baldanti,F., and Sarasini,A. (1994). Comparative quantitation of human cytomegalovirus DNA in blood leukocytes and plasma of transplant and AIDS patients. J. Clin Microbiol. *32*, 2709-2717.

Gerna,G., Lilleri,D., Zecca,M., Alessandrino,E.P., Baldanti,F., Revello,M.G., and Locatelli,F. (2005). Rising antigenemia levels may be misleading in pre-

emptive therapy of human cytomegalovirus infection in allogeneic hematopoietic stem cell transplant recipients. Haematologica *90*, 526-533.

Gerna,G., Percivalle,E., Baldanti,F., Sozzani,S., Lanzarini,P., Genini,E., Lilleri,D., and Revello,M.G. (2000). Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. J. Virol. *74*, 5629-5638.

Gerna,G., Zipeto,D., Percivalle,E., Parea,M., Revello,M.G., Maccario,R., Peri,G., and Milanesi,G. (1992). Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. J. Infect. Dis. *166*, 1236-1244.

Gilbert,M.J., Riddell,S.R., Plachter,B., and Greenberg,P.D. (1996). Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature *383*, 720-722.

Gillespie,G.M., Wills,M.R., Appay,V., O'Callaghan,C., Murphy,M., Smith,N., Sissons,P., Rowland-Jones,S., Bell,J.I., and Moss,P.A. (2000). Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. J. Virol. *74*, 8140-8150.

Giralt,S., Estey,E., Albitar,M., van,B.K., Rondon,G., Anderlini,P., O'Brien,S., Khouri,I., Gajewski,J., Mehra,R., Claxton,D., Andersson,B., Beran,M., Przepiorka,D., Koller,C., Kornblau,S., Korbling,M., Keating,M., Kantarjian,H., and Champlin,R. (1997). Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. Blood *89*, 4531-4536.

Giraud,G., Bogdanovic,G., Priftakis,P., Remberger,M., Svahn,B.M., Barkholt,L., Ringden,O., Winiarski,J., Ljungman,P., and Dalianis,T. (2006). The incidence of hemorrhagic cystitis and BK-viruria in allogeneic hematopoietic stem cell recipients according to intensity of the conditioning regimen 14. Haematologica *91*, 401-404.

Glucksberg,H., Storb,R., Fefer,A., Buckner,C.D., Neiman,P.E., Clift,R.A., Lerner,K.G., and Thomas,E.D. (1974). Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation *18*, 295-304.

Goldman,J.M., Gale,R.P., Horowitz,M.M., Biggs,J.C., Champlin,R.E., Gluckman,E., Hoffmann,R.G., Jacobsen,S.J., Marmont,A.M., McGlave,P.B., and . (1988). Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Ann Intern Med *108*, 806-814.

Goodpasture,E.W. and Talbot,F.B. (1921). Concerning the nature of 'protozoan-like' cells in certain lesions of infancy. Am J Dis Child *21*, 415-425.

Goodrich,J.M., Bowden,R.A., Fisher,L., Keller,C., Schoch,G., and Meyers,J.D. (1993). Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. Ann Intern Med *118*, 173-178.

Goodrich,J.M., Mori,M., Gleaves,C.A., Du,M.C., Cays,M., Ebeling,D.F., Buhles,W.C., DeArmond,B., and Meyers,J.D. (1991). Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. N. Engl. J. Med *325*, 1601-1607.

Gopal,I.N., Quinn,A., Henry,S.C., Hamilton,J.D., Staats,H.F., and Frothingham,R. (2005). Nasal peptide vaccination elicits CD8 responses and reduces viral burden after challenge with virulent murine cytomegalovirus 2. Microbiol. Immunol. *49*, 113-119.

Gor,D., Sabin,C., Prentice,H.G., Vyas,N., Man,S., Griffiths,P.D., and Emery,V.C. (1998). Longitudinal fluctuations in cytomegalovirus load in bone marrow transplant patients: relationship between peak virus load, donor/recipient serostatus, acute GVHD and CMV disease. Bone Marrow Transplant. *21*, 597-605.

Gorin,N.C., Labopin,M., Rocha,V., Arcese,W., Beksac,M., Gluckman,E., Ringden,O., Ruutu,T., Reiffers,J., Bandini,G., Falda,M., Zikos,P., Willemze,R., and Frassoni,F. (2003). Marrow versus peripheral blood for geno-identical allogeneic stem cell transplantation in acute myelocytic leukemia: influence of dose and stem cell source shows better outcome with rich marrow. Blood *102*, 3043-3051.

Gratama,J.W., van Esser,J.W., Lamers,C.H., Tournay,C., Lowenberg,B., Bolhuis,R.L., and Cornelissen,J.J. (2001). Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. Blood *98*, 1358-1364.

Gratwohl,A., Baldomero,H., Frauendorfer,K., and Urbano-Ispizua,A. (2006a). EBMT activity survey 2004 and changes in disease indication over the past 15 years. Bone Marrow Transplant. *37*, 1069-1085.

Gratwohl,A., Brand,R., Apperley,J., Crawley,C., Ruutu,T., Corradini,P., Carreras,E., Devergie,A., Guglielmi,C., Kolb,H.J., and Niederwieser,D. (2006b). Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). Haematologica *91*, 513-521.

Gratwohl,A., Brand,R., Frassoni,F., Rocha,V., Niederwieser,D., Reusser,P., Einsele,H., and Cordonnier,C. (2005). Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time 24. Bone Marrow Transplant. *36*, 757-769.

Graw,R.G., Jr., Lohrmann,H.P., Bull,M.I., Decter,J., Herzig,G.P., Bull,J.M., Leventhal,B.G., Yankee,R.A., Herzig,R.H., Krueger,G.R., Bleyer,W.A., Buja,M.L., McGinniss,M.H., Alter,H.J., Whang-Peng,J., Gralnick,H.R., Kirkpatrick,C.H., and Henderson,E.S. (1974). Bone-marrow transplantation following combination chemotherapy immunosuppression (B.A.C.T.) in patients with acute leukemia. Transplant. Proc. *6*, 349-354.

Gredmark,S., Tilburgs,T., and Soderberg-Naucler,C. (2004). Human cytomegalovirus inhibits cytokine-induced macrophage differentiation. J Virol *78*, 10378-10389.

Grefte,A., Harmsen,M.C., van der,G.M., Knollema,S., van Son,W.J., and The,T.H. (1994). Presence of human cytomegalovirus (HCMV) immediate early mRNA but not ppUL83 (lower matrix protein pp65) mRNA in polymorphonuclear and mononuclear leukocytes during active HCMV infection. J. Gen. Virol. *75 (Pt 8)*, 1989-1998.

Griffiths,P.D. and Baboonian,C. (1984). A prospective study of primary cytomegalovirus infection during pregnancy: final report. Br. J. Obstet. Gynaecol. *91*, 307-315.

Griffiths,P.D., Panjwani,D.D., Stirk,P.R., Ball,M.G., Ganczakowski,M., Blacklock,H.A., and Prentice,H.G. (1984). Rapid diagnosis of cytomegalovirus infection in immunocompromised patients by detection of early antigen fluorescent foci. Lancet *2*, 1242-1245.

Grob,J.P., Grundy,J.E., Prentice,H.G., Griffiths,P.D., Hoffbrand,A.V., Hughes,M.D., Tate,T., Wimperis,J.Z., and Brenner,M.K. (1987). Immune donors can protect marrow-transplant recipients from severe cytomegalovirus infections. Lancet *1*, 774-776.

Grundy,J.E., Shanley,J.D., and Griffiths,P.D. (1987). Is cytomegalovirus interstitial pneumonitis in transplant recipients an immunopathological condition? Lancet *2*, 996-999.

Guglielmi,C., Arcese,W., Dazzi,F., Brand,R., Bunjes,D., Verdonck,L.F., Schattenberg,A., Kolb,H.J., Ljungman,P., Devergie,A., Bacigalupo,A., Gomez,M., Michallet,M., Elmaagacli,A., Gratwohl,A., Apperley,J., and Niederwieser,D. (2002). Donor lymphocyte infusion for relapsed chronic myelogenous leukemia: prognostic relevance of the initial cell dose. Blood *100*, 397-405.

Guild,W.R., Harrison,J.H., Merrill,J.P., and Murray,J. (1955). Successful homotransplantation of the kidney in an identical twin. Trans. Am. Clin Climatol. Assoc. *67*, 167-173.

Gyulai,Z., Endresz,V., Burian,K., Pincus,S., Toldy,J., Cox,W.I., Meric,C., Plotkin,S., Gonczol,E., and Berencsi,K. (2000). Cytotoxic T lymphocyte (CTL) responses to human cytomegalovirus pp65, IE1-Exon4, gB, pp150, and pp28 in healthy individuals: reevaluation of prevalence of IE1-specific CTLs. J Infect. Dis *181*, 1537-1546.

Hahn,G., Jores,R., and Mocarski,E.S. (1998). Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc. Natl. Acad. Sci. U. S. A *95*, 3937-3942.

Hahn,T., Wall,D., Camitta,B., Davies,S., Dillon,H., Gaynon,P., Larson,R.A., Parsons,S., Seidenfeld,J., Weisdorf,D., and McCarthy,P.L., Jr. (2006). The role of cytotoxic therapy with hematopoietic stem cell transplantation in the therapy of acute lymphoblastic leukemia in adults: an evidence-based review. Biol. Blood Marrow Transplant. *12*, 1-30.

Hakki,M., Riddell,S.R., Storek,J., Carter,R.A., Stevens-Ayers,T., Sudour,P., White,K., Corey,L., and Boeckh,M. (2003). Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. Blood *102*, 3060-3067.

Hale,G., Bright,S., Chumbley,G., Hoang,T., Metcalf,D., Munro,A.J., and Waldmann,H. (1983). Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. Blood *62*, 873-882.

Hale,G., Zhang,M.J., Bunjes,D., Prentice,H.G., Spence,D., Horowitz,M.M., Barrett,A.J., and Waldmann,H. (1998). Improving the outcome of bone marrow transplantation by using CD52 monoclonal antibodies to prevent graft-versus-host disease and graft rejection. Blood *92*, 4581-4590.

Hamaki,T., Kami,M., Kanda,Y., Yuji,K., Inamoto,Y., Kishi,Y., Nakai,K., Nakayama,I., Murashige,N., Abe,Y., Ueda,Y., Hino,M., Inoue,T., Ago,H., Hidaka,M., Hayashi,T., Yamane,T., Uoshima,N., Miyakoshi,S., and Taniguchi,S. (2005). Reduced-intensity stem-cell transplantation for adult acute lymphoblastic leukemia: a retrospective study of 33 patients. Bone Marrow Transplant. *35*, 549-556.

Handsfield,H.H., Chandler,S.H., Caine,V.A., Meyers,J.D., Corey,L., Medeiros,E., and McDougall,J.K. (1985). Cytomegalovirus infection in sex partners: evidence for sexual transmission. J. Infect. Dis. *151*, 344-348.

Hansen,J.A., Gooley,T.A., Martin,P.J., Appelbaum,F., Chauncey,T.R., Clift,R.A., Petersdorf,E.W., Radich,J., Sanders,J.E., Storb,R.F., Sullivan,K.M., and Anasetti,C. (1998). Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. N. Engl. J. Med *338*, 962-968.

Hassan-Walker,A.F., Kidd,I.M., Sabin,C., Sweny,P., Griffiths,P.D., and Emery,V.C. (1999). Quantity of human cytomegalovirus (CMV) DNAemia as a risk factor for CMV disease in renal allograft recipients: relationship with donor/recipient CMV serostatus, receipt of augmented methylprednisolone and antithymocyte globulin (ATG). J. Med. Virol. *58*, 182-187.

Hayashi,M.L., Blankenship,C., and Shenk,T. (2000). Human cytomegalovirus UL69 protein is required for efficient accumulation of infected cells in the G1 phase of the cell cycle. Proc. Natl. Acad. Sci. U. S. A *97*, 2692-2696.

He,H., Rinaldo,C.R., Jr., and Morel,P.A. (1995). T cell proliferative responses to five human cytomegalovirus proteins in healthy seropositive individuals: implications for vaccine development. J. Gen. Virol. *76 (Pt 7)*, 1603-1610.

He,R., Ruan,Q., Qi,Y., Ma,Y.P., Huang,Y.J., Sun,Z.R., and Ji,Y.H. (2006). Sequence variability of human cytomegalovirus UL146 and UL147 genes in low-passage clinical isolates. Intervirology *49*, 215-223.

Hebart,H., Muller,C., Loffler,J., Jahn,G., and Einsele,H. (1996). Monitoring of CMV infection: a comparison of PCR from whole blood, plasma-PCR, pp65-antigenemia and virus culture in patients after bone marrow transplantation. Bone Marrow Transplant. *17*, 861-868.

Hegde,N.R., Tomazin,R.A., Wisner,T.W., Dunn,C., Boname,J.M., Lewinsohn,D.M., and Johnson,D.C. (2002). Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. J Virol *76*, 10929-10941.

Hendrix,M.G., Salimans,M.M., van Boven,C.P., and Bruggeman,C.A. (1990). High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. Am J Pathol. *136*, 23-28.

Herr,A.L., Labopin,M., Blaise,D., Milpied,N., Potter,M., Michallet,M., Heit,W., Ferrara,F., Esteve,J., Arcese,W., Ehninger,G., Rowe,J.M., Kobbe,G., Rosselet,A., Bunjes,D., Rio,B., Brune,M., Nagler,A., Gorin,N.C., Frassoni,F., and Rocha,V. (2007). HLA-identical sibling allogeneic peripheral blood stem cell transplantation with reduced intensity conditioning compared to autologous peripheral blood stem cell transplantation for elderly patients with de novo acute myeloid leukemia. Leukemia *21*, 129-135.

Hertenstein,B., Hampl,W., Bunjes,D., Wiesneth,M., Duncker,C., Koszinowski,U., Heimpel,H., Arnold,R., and Mertens,T. (1995). In vivo/ex vivo T cell depletion for GVHD prophylaxis influences onset and course of active cytomegalovirus infection and disease after BMT. Bone Marrow Transplant. *15*, 387-393.

Heymann,J.B., Cheng,N., Newcomb,W.W., Trus,B.L., Brown,J.C., and Steven,A.C. (2003). Dynamics of herpes simplex virus capsid maturation visualized by time-lapse cryo-electron microscopy. Nat. Struct. Biol. *10*, 334-341.

Ho,S.K., Lo,C.Y., Cheng,I.K., and Chan,T.M. (1998). Rapid cytomegalovirus pp65 antigenemia assay by direct erythrocyte lysis and immunofluorescence staining. J. Clin Microbiol. *36*, 638-640.

Hobom,U., Brune,W., Messerle,M., Hahn,G., and Koszinowski,U.H. (2000). Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J Virol *74*, 7720-7729.

Hokland,M., Jacobsen,N., Ellegaard,J., and Hokland,P. (1988). Natural killer function following allogeneic bone marrow transplantation. Very early reemergence but strong dependence of cytomegalovirus infection. Transplantation *45*, 1080-1084.

Howden,B.P., Michaelides,A., Spelman,D.W., Spencer,A., Schwarer,A.P., Wesselingh,S., and Kotsimbos,T.C. (2003). Cytomegalovirus viral load monitoring after allogeneic bone marrow transplantation in patients receiving antiviral prophylaxis. Bone Marrow Transplant. *32*, 795-800.

Huber,M.T. and Compton,T. (1998). The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. J Virol *72*, 8191-8197.

Humar,A., Gregson,D., Caliendo,A.M., McGeer,A., Malkan,G., Krajden,M., Corey,P., Greig,P., Walmsley,S., Levy,G., and Mazzulli,T. (1999). Clinical utility of quantitative cytomegalovirus viral load determination for predicting cytomegalovirus disease in liver transplant recipients. Transplantation *68*, 1305-1311.

Humar,A., Kumar,D., Boivin,G., and Caliendo,A.M. (2002). Cytomegalovirus (CMV) virus load kinetics to predict recurrent disease in solid-organ transplant patients with CMV disease. J Infect. Dis *186*, 829-833.

Hume,D.M., Merrill,J.P., Miller,B.F., and Thorn,G.W. (1955). Experiences with renal homotransplantation in the human: report of nine cases. J. Clin Invest *34*, 327-382.

Husni,R.N., Gordon,S.M., Longworth,D.L., Arroliga,A., Stillwell,P.C., Avery,R.K., Maurer,J.R., Mehta,A., and Kirby,T. (1998). Cytomegalovirus infection is a risk factor for invasive aspergillosis in lung transplant recipients. Clin Infect. Dis. *26*, 753-755.

Ichinohe,T., Uchiyama,T., Shimazaki,C., Matsuo,K., Tamaki,S., Hino,M., Watanabe,A., Hamaguchi,M., Adachi,S., Gondo,H., Uoshima,N., Yoshihara,T., Hatanaka,K., Fujii,H., Kawa,K., Kawanishi,K., Oka,K., Kimura,H., Itoh,M., Inukai,T., Maruya,E., Saji,H., and Kodera,Y. (2004). Feasibility of HLA-haploidentical hematopoietic stem cell transplantation between noninherited maternal antigen (NIMA)-mismatched family members linked with long-term fetomaternal microchimerism. Blood *104*, 3821-3828.

Iwasaki,S., Yamashita,M., Maeda,M., Misawa,K., and Mineta,H. (2007). Audiological outcome of infants with congenital cytomegalovirus infection in a prospective study. Audiol. Neurootol. *12*, 31-36.

Jaboulay, M. Greffe du reins au pli du coude par soudure arte. Bull Lyon Med 107, 575. 1906.
Ref Type: Generic

Jahn,G., Scholl,B.C., Traupe,B., and Fleckenstein,B. (1987). The two major structural phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties. J Gen. Virol *68 (Pt 5)*, 1327-1337.

Jahn,G., Stenglein,S., Riegler,S., Einsele,H., and Sinzger,C. (1999). Human cytomegalovirus infection of immature dendritic cells and macrophages. Intervirology *42*, 365-372.

Janssen,E.M., Lemmens,E.E., Wolfe,T., Christen,U., von Herrath,M.G., and Schoenberger,S.P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature *421*, 852-856.

Jessionek,K. (1904). Ueber einen befund von protozoen-artigen gebilden in den organem eines heretitarluetischen foetus. Munch Med Wochenschr *51*, 1905-1907.

Jiwa,N.M., Van Gemert,G.W., Raap,A.K., Van de Rijke,F.M., Mulder,A., Lens,P.F., Salimans,M.M., Zwaan,F.E., Van,D.W., and Van der,P.M. (1989). Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation *48*, 72-76.

Johnson,F.L., Look,A.T., Gockerman,J., Ruggiero,M.R., la-Pozza,L., and Billings,F.T., III (1984). Bone-marrow transplantation in a patient with sickle-cell anemia. N. Engl. J. Med *311*, 780-783.

Johnson,L.O., Marks,E.K., Robson,M.J., Gaston,E.O., and Zirkle,R.E. (1949). Effect of spleen protection on mortality following X-irradiation. J. Lab Clin Med *34*, 1543-1538.

Jones,R.J., Ambinder,R.F., Piantadosi,S., and Santos,G.W. (1991). Evidence of a graft-versus-lymphoma effect associated with allogeneic bone marrow transplantation. Blood *77*, 649-653.

Jones,T.R., Wiertz,E.J., Sun,L., Fish,K.N., Nelson,J.A., and Ploegh,H.L. (1996). Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. Proc. Natl. Acad. Sci. U. S. A *93*, 11327-11333.

Jonjic,S., Pavic,I., Polic,B., Crnkovic,I., Lucin,P., and Koszinowski,U.H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J. Exp. Med *179*, 1713-1717.

Juliusson,G., Theorin,N., Karlsson,K., Frodin,U., and Malm,C. (2006). Subcutaneous alemtuzumab vs ATG in adjusted conditioning for allogeneic transplantation: influence of Campath dose on lymphoid recovery, mixed chimerism and survival
2. Bone Marrow Transplant. *37*, 503-510.

Junghanss,C., Boeckh,M., Carter,R.A., Sandmaier,B.M., Maris,M.B., Maloney,D.G., Chauncey,T., McSweeney,P.A., Little,M.T., Corey,L., and Storb,R. (2002). Incidence and outcome of cytomegalovirus infections following

nonmyeloablative compared with myeloablative allogeneic stem cell transplantation, a matched control study. Blood *99*, 1978-1985.

Kahl,C., Leisenring,W., Deeg,H.J., Chauncey,T.R., Flowers,M.E., Martin,P.J., Sanders,J.E., and Storb,R. (2005). Cyclophosphamide and antithymocyte globulin as a conditioning regimen for allogeneic marrow transplantation in patients with aplastic anaemia: a long-term follow-up. Br. J. Haematol. *130*, 747-751.

Kalejta,R.F., Bechtel,J.T., and Shenk,T. (2003). Human cytomegalovirus pp71 stimulates cell cycle progression by inducing the proteasome-dependent degradation of the retinoblastoma family of tumor suppressors. Mol. Cell Biol. *23*, 1885-1895.

Kalejta,R.F. and Shenk,T. (2003). The human cytomegalovirus UL82 gene product (pp71) accelerates progression through the G1 phase of the cell cycle. J Virol *77*, 3451-3459.

Kari,B., Liu,Y.N., Goertz,R., Lussenhop,N., Stinski,M.F., and Gehrz,R. (1990). Structure and composition of a family of human cytomegalovirus glycoprotein complexes designated gC-I (gB). J Gen. Virol *71 (Pt 11)*, 2673-2680.

Keating,M.J., O'Brien,S., Kontoyiannis,D., Plunkett,W., Koller,C., Beran,M., Lerner,S., and Kantarjian,H. (2002). Results of first salvage therapy for patients refractory to a fludarabine regimen in chronic lymphocytic leukemia. Leuk. Lymphoma *43*, 1755-1762.

Kern,F., Bunde,T., Faulhaber,N., Kiecker,F., Khatamzas,E., Rudawski,I.M., Pruss,A., Gratama,J.W., Volkmer-Engert,R., Ewert,R., Reinke,P., Volk,H.D., and Picker,L.J. (2002). Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J. Infect. Dis. *185*, 1709-1716.

Kern,F., Surel,I.P., Brock,C., Freistedt,B., Radtke,H., Scheffold,A., Blasczyk,R., Reinke,P., Schneider-Mergener,J., Radbruch,A., Walden,P., and Volk,H.D. (1998). T-cell epitope mapping by flow cytometry
73. Nat. Med *4*, 975-978.

Kern,F., Surel,I.P., Faulhaber,N., Frommel,C., Schneider-Mergener,J., Schonemann,C., Reinke,P., and Volk,H.D. (1999). Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J. Virol. *73*, 8179-8184.

Kernan,N.A., Bartsch,G., Ash,R.C., Beatty,P.G., Champlin,R., Filipovich,A., Gajewski,J., Hansen,J.A., Henslee-Downey,J., McCullough,J., and . (1993). Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. N. Engl. J. Med *328*, 593-602.

Khan,N., Cobbold,M., Keenan,R., and Moss,P.A. (2002a). Comparative analysis of CD8+ T cell responses against human cytomegalovirus proteins pp65 and

immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. J. Infect. Dis. *185*, 1025-1034.

Khan,N., Shariff,N., Cobbold,M., Bruton,R., Ainsworth,J.A., Sinclair,A.J., Nayak,L., and Moss,P.A. (2002b). Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol. *169*, 1984-1992.

Khattab,B.A., Lindenmaier,W., Frank,R., and Link,H. (1997). Three T-cell epitopes within the C-terminal 265 amino acids of the matrix protein pp65 of human cytomegalovirus recognized by human lymphocytes. J. Med. Virol. *52*, 68-76.

Khouri,I.F., Keating,M.J., Saliba,R.M., and Champlin,R.E. (2002). Long-term follow-up of patients with CLL treated with allogeneic hematopoietic transplantation. Cytotherapy. *4*, 217-221.

Khouri,I.F., Lee,M.S., Saliba,R.M., Andersson,B., Anderlini,P., Couriel,D., Hosing,C., Giralt,S., Korbling,M., McMannis,J., Keating,M.J., and Champlin,R.E. (2004). Nonablative allogeneic stem cell transplantation for chronic lymphocytic leukemia: impact of rituximab on immunomodulation and survival. Exp. Hematol. *32*, 28-35.

Khouri,I.F., Przepiorka,D., van,B.K., O'Brien,S., Palmer,J.L., Lerner,S., Mehra,R.C., Vriesendorp,H.M., Andersson,B.S., Giralt,S., Korbling,M., Keating,M.J., and Champlin,R.E. (1997). Allogeneic blood or marrow transplantation for chronic lymphocytic leukaemia: timing of transplantation and potential effect of fludarabine on acute graft-versus-host disease. Br. J. Haematol. *97*, 466-473.

Khouri,I.F., Saliba,R.M., Giralt,S.A., Lee,M.S., Okoroji,G.J., Hagemeister,F.B., Korbling,M., Younes,A., Ippoliti,C., Gajewski,J.L., McLaughlin,P., Anderlini,P., Donato,M.L., Cabanillas,F.F., and Champlin,R.E. (2001). Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. Blood *98*, 3595-3599.

Kidd,I.M., Fox,J.C., Pillay,D., Charman,H., Griffiths,P.D., and Emery,V.C. (1993). Provision of prognostic information in immunocompromised patients by routine application of the polymerase chain reaction for cytomegalovirus. Transplantation *56*, 867-871.

Kinch,A., Oberg,G., Arvidson,J., Falk,K.I., Linde,A., and Pauksens,K. (2007). Post-transplant lymphoproliferative disease and other Epstein-Barr virus diseases in allogeneic haematopoietic stem cell transplantation after introduction of monitoring of viral load by polymerase chain reaction
6. Scand. J. Infect. Dis. *39*, 235-244.

Klangsinsirikul,P., Carter,G.I., Byrne,J.L., Hale,G., and Russell,N.H. (2002). Campath-1G causes rapid depletion of circulating host dendritic cells (DCs)

before allogeneic transplantation but does not delay donor DC reconstitution. Blood *99*, 2586-2591.

Kline,J., Pollyea,D.A., Stock,W., Artz,A., Rich,E., Godley,L., Zimmerman,T., Thompson,K., Pursell,K., Larson,R.A., and van,B.K. (2006). Pre-transplant ganciclovir and post transplant high-dose valacyclovir reduce CMV infections after alemtuzumab-based conditioning. Bone Marrow Transplant. *37*, 307-310.

Kocher,A.A., Bonaros,N., Dunkler,D., Ehrlich,M., Schlechta,B., Zweytick,B., Grimm,M., Zuckermann,A., Wolner,E., and Laufer,G. (2003). Long-term results of CMV hyperimmune globulin prophylaxis in 377 heart transplant recipients. J Heart Lung Transplant. *22*, 250-257.

Kojima,S., Inaba,J., Yoshimi,A., Takahashi,Y., Watanabe,N., Kudo,K., Horibe,K., Maeda,N., Kato,K., and Matsuyama,T. (2001). Unrelated donor marrow transplantation in children with severe aplastic anaemia using cyclophosphamide, anti-thymocyte globulin and total body irradiation. Br. J. Haematol. *114*, 706-711.

Kolb,H.J., Mittermuller,J., Clemm,C., Holler,E., Ledderose,G., Brehm,G., Heim,M., and Wilmanns,W. (1990). Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood *76*, 2462-2465.

Kollert-Jons,A., Bogner,E., and Radsak,K. (1991). A 15-kilobase-pair region of the human cytomegalovirus genome which includes US1 through US13 is dispensable for growth in cell culture. J. Virol. *65*, 5184-5189.

Komanduri,K.V., Viswanathan,M.N., Wieder,E.D., Schmidt,D.K., Bredt,B.M., Jacobson,M.A., and McCune,J.M. (1998). Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nat. Med *4*, 953-956.

Kondo,K., Xu,J., and Mocarski,E.S. (1996). Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proc. Natl. Acad. Sci. U. S. A *93*, 11137-11142.

Konoplev,S., Champlin,R.E., Giralt,S., Ueno,N.T., Khouri,I., Raad,I., Rolston,K., Jacobson,K., Tarrand,J., Luna,M., Nguyen,Q., and Whimbey,E. (2001). Cytomegalovirus pneumonia in adult autologous blood and marrow transplant recipients. Bone Marrow Transplant. *27*, 877-881.

Kook,H., Goldman,F., Padley,D., Giller,R., Rumelhart,S., Holida,M., Lee,N., Peters,C., Comito,M., Huling,D., and Trigg,M. (1996). Reconstruction of the immune system after unrelated or partially matched T-cell-depleted bone marrow transplantation in children: immunophenotypic analysis and factors affecting the speed of recovery. Blood *88*, 1089-1097.

Kotenko,S.V., Saccani,S., Izotova,L.S., Mirochnitchenko,O.V., and Pestka,S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc. Natl. Acad. Sci. U. S. A *97*, 1695-1700.

Kottaridis,P.D., Milligan,D.W., Chopra,R., Chakraverty,R.K., Chakrabarti,S., Robinson,S., Peggs,K., Verfuerth,S., Pettengell,R., Marsh,J.C., Schey,S., Mahendra,P., Morgan,G.J., Hale,G., Waldmann,H., de Elvira,M.C., Williams,C.D., Devereux,S., Linch,D.C., Goldstone,A.H., and Mackinnon,S. (2000). In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation. Blood *96*, 2419-2425.

Kroger,N., Zabelina,T., Kruger,W., Renges,H., Stute,N., Schrum,J., Kabisch,H., Schafhausen,P., Jaburg,N., Loliger,C., Schafer,P., Hinke,A., and Zander,A.R. (2001). Patient cytomegalovirus seropositivity with or without reactivation is the most important prognostic factor for survival and treatment-related mortality in stem cell transplantation from unrelated donors using pretransplant in vivo T-cell depletion with anti-thymocyte globulin. Br. J Haematol. *113*, 1060-1071.

Kroger,N., Zabelina,T., Schieder,H., Panse,J., Ayuk,F., Stute,N., Fehse,N., Waschke,O., Fehse,B., Kvasnicka,H.M., Thiele,J., and Zander,A. (2005). Pilot study of reduced-intensity conditioning followed by allogeneic stem cell transplantation from related and unrelated donors in patients with myelofibrosis. Br. J. Haematol. *128*, 690-697.

Kruger,R.M., Paranjothi,S., Storch,G.A., Lynch,J.P., and Trulock,E.P. (2003). Impact of prophylaxis with cytogam alone on the incidence of CMV viremia in CMV-seropositive lung transplant recipients. J Heart Lung Transplant. *22*, 754-763.

La Rosa,C., Wang,Z., Lacey,S.F., Markel,S.F., Sharma,M.C., Martinez,J., Lalimarmo,M.M., and Diamond,D.J. (2005). Characterization of host immunity to cytomegalovirus pp150 (UL32). Hum. Immunol. *66*, 116-126.

Labar,B., Suciu,S., Zittoun,R., Muus,P., Marie,J.P., Fillet,G., Peetermans,M., Stryckmans,P., Willemze,R., Feremans,W., Jaksic,B., Bourhis,J.H., Burghouts,J.P., and De,W.T. (2004). Allogeneic stem cell transplantation in acute lymphoblastic leukemia and non-Hodgkin's lymphoma for patients <or=50 years old in first complete remission: results of the EORTC ALL-3 trial. Haematologica *89*, 809-817.

Lamba,R., Carrum,G., Myers,G.D., Bollard,C.M., Krance,R.A., Heslop,H.E., Brenner,M.K., and Popat,U. (2005). Cytomegalovirus (CMV) infections and CMV-specific cellular immune reconstitution following reduced intensity conditioning allogeneic stem cell transplantation with Alemtuzumab. Bone Marrow Transplant. *36*, 797-802.

Lang,D.J., Scolnick,E.M., and Willerson,J.T. (1968). Association of cytomegalovirus infection with the postperfusion syndrome. N. Engl. J Med *278*, 1147-1149.

Larsen,M.V., Lundegaard,C., Lamberth,K., Buus,S., Brunak,S., Lund,O., and Nielsen,M. (2005). An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. Eur. J. Immunol. *35*, 2295-2303.

Larsson,K., Lonnqvist,B., Ringden,O., Hedquist,B., and Ljungman,P. (2002). CMV retinitis after allogeneic bone marrow transplantation: a report of five cases 6. Transpl. Infect. Dis. *4*, 75-79.

Larsson,S., Soderberg-Naucler,C., Wang,F.Z., and Moller,E. (1998). Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. Transfusion *38*, 271-278.

Laughlin-Taylor,E., Pande,H., Forman,S.J., Tanamachi,B., Li,C.R., Zaia,J.A., Greenberg,P.D., and Riddell,S.R. (1994). Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J. Med Virol. *43*, 103-110.

Li Pira,G., Bottone,L., Ivaldi,F., Pelizzoli,R., Bracci,L., Lozzi,L., Scarso,L., Tripodi,G., and Manca,F. (2004a). Recognition of CMV pp65 protein antigen by human CD4 T-cell lines induced with an immunodominant peptide pool. Hum. Immunol. *65*, 537-543.

Li Pira,G., Bottone,L., Ivaldi,F., Pelizzoli,R., Del,G.F., Lozzi,L., Bracci,L., Loregian,A., Palu,G., De,P.R., Einsele,H., and Manca,F. (2004b). Identification of new Th peptides from the cytomegalovirus protein pp65 to design a peptide library for generation of CD4 T cell lines for cellular immunoreconstitution. Int. Immunol. *16*, 635-642.

Li,C.R., Greenberg,P.D., Gilbert,M.J., Goodrich,J.M., and Riddell,S.R. (1994). Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood *83*, 1971-1979.

Li,L., Nelson,J.A., and Britt,W.J. (1997). Glycoprotein H-related complexes of human cytomegalovirus: identification of a third protein in the gCIII complex. J Virol *71*, 3090-3097.

Lipson,S.M., Shepp,D.H., Match,M.E., Axelrod,F.B., and Whitbread,J.A. (2001). Cytomegalovirus infectivity in whole blood following leukocyte reduction by filtration. Am J Clin Pathol. *116*, 52-55.

Littler,E., Stuart,A.D., and Chee,M.S. (1992). Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature *358*, 160-162.

Liu,Y.N., Curtsinger,J., Donahue,P.R., Klaus,A., Optiz,G., Cooper,J., Karr,R.W., Bach,F.H., and Gehrz,R.C. (1993). Molecular analysis of the immune response to human cytomegalovirus glycoprotein B. I. Mapping of HLA-restricted helper T cell epitopes on gp93. J. Gen. Virol. *74 (Pt 10)*, 2207-2214.

Liu,Z., Savoldo,B., Huls,H., Lopez,T., Gee,A., Wilson,J., Brenner,M.K., Heslop,H.E., and Rooney,C.M. (2002). Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the prevention and treatment of EBV-associated

post-transplant lymphomas
3. Recent Results Cancer Res. *159*, 123-133.

Ljungman,P. (1995). Cytomegalovirus pneumonia: presentation, diagnosis, and treatment. Semin. Respir. Infect. *10*, 209-215.

Ljungman,P., Brand,R., Einsele,H., Frassoni,F., Niederwieser,D., and Cordonnier,C. (2003a). Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. Blood *102*, 4255-4260.

Ljungman,P., de La,C.R., Milpied,N., Volin,L., Russell,C.A., Crisp,A., and Webster,A. (2002a). Randomized study of valacyclovir as prophylaxis against cytomegalovirus reactivation in recipients of allogeneic bone marrow transplants. Blood *99*, 3050-3056.

Ljungman,P., Deliliers,G.L., Platzbecker,U., Matthes-Martin,S., Bacigalupo,A., Einsele,H., Ullmann,J., Musso,M., Trenschel,R., Ribaud,P., Bornhauser,M., Cesaro,S., Crooks,B., Dekker,A., Gratecos,N., Klingebiel,T., Tagliaferri,E., Ullmann,A.J., Wacker,P., and Cordonnier,C. (2001a). Cidofovir for cytomegalovirus infection and disease in allogeneic stem cell transplant recipients. The Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Blood *97*, 388-392.

Ljungman,P., Engelhard,D., Link,H., Biron,P., Brandt,L., Brunet,S., Cordonnier,C., Debusscher,L., de,L.A., Kolb,H.J., and . (1992). Treatment of interstitial pneumonitis due to cytomegalovirus with ganciclovir and intravenous immune globulin: experience of European Bone Marrow Transplant Group. Clin Infect. Dis. *14*, 831-835.

Ljungman,P., Griffiths,P., and Paya,C. (2002b). Definitions of cytomegalovirus infection and disease in transplant recipients. Clin Infect. Dis *34*, 1094-1097.

Ljungman,P., Lore,K., Aschan,J., Klaesson,S., Lewensohn-Fuchs,I., Lonnqvist,B., Ringden,O., Winiarski,J., and Ehrnst,A. (1996a). Use of a semi-quantitative PCR for cytomegalovirus DNA as a basis for pre-emptive antiviral therapy in allogeneic bone marrow transplant patients. Bone Marrow Transplant. *17*, 583-587.

Ljungman,P., Oberg,G., Aschan,J., Ehrnst,A., Lonnqvist,B., Pauksen,K., and Sulila,P. (1996b). Foscarnet for pre-emptive therapy of CMV infection detected by a leukocyte-based nested PCR in allogeneic bone marrow transplant patients. Bone Marrow Transplant. *18*, 565-568.

Ljungman,P., Perez-Bercoff,L., Jonsson,J., Avetisyan,G., Sparrelid,E., Aschan,J., Barkholt,L., Larsson,K., Winiarski,J., Yun,Z., and Ringden,O. (2006). Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation. Haematologica *91*, 78-83.

Ljungman,P., Ribaud,P., Eyrich,M., Matthes-Martin,S., Einsele,H., Bleakley,M., Machaczka,M., Bierings,M., Bosi,A., Gratecos,N., and Cordonnier,C. (2003b).

Cidofovir for adenovirus infections after allogeneic hematopoietic stem cell transplantation: a survey by the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation
22. Bone Marrow Transplant. *31*, 481-486.

Ljungman,P., Wang,F.Z., Clark,D.A., Emery,V.C., Remberger,M., Ringden,O., and Linde,A. (2000). High levels of human herpesvirus 6 DNA in peripheral blood leucocytes are correlated to platelet engraftment and disease in allogeneic stem cell transplant patients. Br. J Haematol. *111*, 774-781.

Ljungman,P., Ward,K.N., Crooks,B.N., Parker,A., Martino,R., Shaw,P.J., Brinch,L., Brune,M., de La,C.R., Dekker,A., Pauksen,K., Russell,N., Schwarer,A.P., and Cordonnier,C. (2001b). Respiratory virus infections after stem cell transplantation: a prospective study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation
5. Bone Marrow Transplant. *28*, 479-484.

Locatelli,F., Rocha,V., Reed,W., Bernaudin,F., Ertem,M., Grafakos,S., Brichard,B., Li,X., Nagler,A., Giorgiani,G., Haut,P.R., Brochstein,J.A., Nugent,D.J., Blatt,J., Woodard,P., Kurtzberg,J., Rubin,C.M., Miniero,R., Lutz,P., Raja,T., Roberts,I., Will,A.M., Yaniv,I., Vermylen,C., Tannoia,N., Garnier,F., Ionescu,I., Walters,M.C., Lubin,B.H., and Gluckman,E. (2003). Related umbilical cord blood transplantation in patients with thalassemia and sickle cell disease. Blood *101*, 2137-2143.

Loren,A.W., Porter,D.L., Stadtmauer,E.A., and Tsai,D.E. (2003). Post-transplant lymphoproliferative disorder: a review
1. Bone Marrow Transplant. *31*, 145-155.

Lorenz,E., Uphoff,D., Reid,T.R., and Shelton,E. (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer Inst. *12*, 197-201.

Loveland,A.N., Nguyen,N.L., Brignole,E.J., and Gibson,W. (2007). The amino-conserved domain of human cytomegalovirus UL80a proteins is required for key interactions during early stages of capsid formation and virus production. J Virol *81*, 620-628.

Lowance,D., Neumayer,H.H., Legendre,C.M., Squifflet,J.P., Kovarik,J., Brennan,P.J., Norman,D., Mendez,R., Keating,M.R., Coggon,G.L., Crisp,A., and Lee,I.C. (1999). Valacyclovir for the prevention of cytomegalovirus disease after renal transplantation. International Valacyclovir Cytomegalovirus Prophylaxis Transplantation Study Group. N. Engl. J. Med *340*, 1462-1470.

Lu,M. and Shenk,T. (1999). Human cytomegalovirus UL69 protein induces cells to accumulate in G1 phase of the cell cycle. J Virol *73*, 676-683.

Lucarelli,G., Galimberti,M., Polchi,P., Angelucci,E., Baronciani,D., Giardini,C., Politi,P., Durazzi,S.M., Muretto,P., and Albertini,F. (1990). Bone marrow transplantation in patients with thalassemia. N. Engl. J. Med *322*, 417-421.

Lucarelli,G., Polchi,P., Izzi,T., Manna,M., Agostinelli,F., Delfini,C., Porcellini,A., Galimberti,M., Moretti,L., Manna,A., and . (1984). Allogeneic marrow transplantation for thalassemia. Exp. Hematol. *12*, 676-681.

Lucas,K.G., Small,T.N., Heller,G., Dupont,B., and O'Reilly,R.J. (1996). The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. Blood *87*, 2594-2603.

Lucas,M., Day,C.L., Wyer,J.R., Cunliffe,S.L., Loughry,A., McMichael,A.J., and Klenerman,P. (2004). Ex vivo phenotype and frequency of influenza virus-specific CD4 memory T cells. J. Virol. *78*, 7284-7287.

Lurain,N.S., Fox,A.M., Lichy,H.M., Bhorade,S.M., Ware,C.F., Huang,D.D., Kwan,S.P., Garrity,E.R., and Chou,S. (2006). Analysis of the human cytomegalovirus genomic region from UL146 through UL147A reveals sequence hypervariability, genotypic stability, and overlapping transcripts. Virol J *3*, 4.

Lutz,E., Ward,K.N., Szydlo,R., and Goldman,J.M. (1996). Cytomegalovirus antibody avidity in allogeneic bone marrow recipients: evidence for primary or secondary humoral responses depending on donor immune status. J. Med Virol. *49*, 61-65.

MacCormac,L.P. and Grundy,J.E. (1999). Two clinical isolates and the Toledo strain of cytomegalovirus contain endothelial cell tropic variants that are not present in the AD169, Towne, or Davis strains. J Med Virol *57*, 298-307.

MacGregor,R.R., Pakola,S.J., Graziani,A.L., Montzka,D.P., Hodinka,R.L., Nichols,C.W., and Friedman,H.M. (1995). Evidence of active cytomegalovirus infection in clinically stable HIV-infected individuals with CD4+ lymphocyte counts below 100/microliters of blood: features and relation to risk of subsequent CMV retinitis. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. *10*, 324-330.

Mach,M., Kropff,B., Dal,M.P., and Britt,W. (2000). Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). J Virol *74*, 11881-11892.

Mackinnon,S., Barnett,L., Heller,G., and O'Reilly,R.J. (1994). Minimal residual disease is more common in patients who have mixed T-cell chimerism after bone marrow transplantation for chronic myelogenous leukemia. Blood *83*, 3409-3416.

Main, J. M. and Prehn, R. T. Successful skin homografts after the administration of high dosage X radiation with homologous bone marrow. J.Natl.Cancer Inst. 15, 1023-1029. 1955.
Ref Type: Generic

Majhail,N.S., Brunstein,C.G., and Wagner,J.E. (2006). Double umbilical cord blood transplantation. Curr. Opin. Immunol. *18*, 571-575.

Mannick,J.A., Lochte,H.L., Jr., Ashley,C.A., Thomas,E.D., and Ferrebee,J.W. (1960). Autografts of bone marrow in dogs after lethal total-body irradiation. Blood *15*, 255-266.

Marks,D.I., Lush,R., Cavenagh,J., Milligan,D.W., Schey,S., Parker,A., Clark,F.J., Hunt,L., Yin,J., Fuller,S., Vandenberghe,E., Marsh,J., Littlewood,T., Smith,G.M., Culligan,D., Hunter,A., Chopra,R., Davies,A., Towlson,K., and Williams,C.D. (2002). The toxicity and efficacy of donor lymphocyte infusions given after reduced-intensity conditioning allogeneic stem cell transplantation. Blood *100*, 3108-3114.

Marmont,A.M., Horowitz,M.M., Gale,R.P., Sobocinski,K., Ash,R.C., van Bekkum,D.W., Champlin,R.E., Dicke,K.A., Goldman,J.M., Good,R.A., and . (1991). T-cell depletion of HLA-identical transplants in leukemia. Blood *78*, 2120-2130.

Marr,K.A., Carter,R.A., Boeckh,M., Martin,P., and Corey,L. (2002). Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors
5. Blood *100*, 4358-4366.

Martin,P.J., Hansen,J.A., Buckner,C.D., Sanders,J.E., Deeg,H.J., Stewart,P., Appelbaum,F.R., Clift,R., Fefer,A., Witherspoon,R.P., and . (1985). Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. Blood *66*, 664-672.

Mathe,G., Amiel,J.L., Schwarzenberg,L., Cattan,A., and Schneider,M. (1963). Haematopoietic chimera in man after allogenic (homolohos) bone-marrow transplantation. (Control of the secondary syndrome. Specific tolerance due to the chimerism). Br. Med J. *2*, 1633-1635.

Mathe,G., Jammet,H., Pendic,B., Schwarzenberg,L., Duplan,J.F., Maupin,B., Latarjet,R., Larrieu,M.J., Kalic,D., and Djukic,Z. (1959). [Transfusions and grafts of homologous bone marrow in humans after accidental high dose irradiation]. Rev. Fr. Etud. Clin Biol *4*, 226-238.

Matloubian,M., Concepcion,R.J., and Ahmed,R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J. Virol. *68*, 8056-8063.

Mattes,F.M., Hainsworth,E.G., Geretti,A.M., Nebbia,G., Prentice,G., Potter,M., Burroughs,A.K., Sweny,P., Hassan-Walker,A.F., Okwuadi,S., Sabin,C., Amooty,G., Brown,V.S., Grace,S.C., Emery,V.C., and Griffiths,P.D. (2004). A randomized, controlled trial comparing ganciclovir to ganciclovir plus foscarnet (each at half dose) for preemptive therapy of cytomegalovirus infection in transplant recipients. J. Infect. Dis. *189*, 1355-1361.

Mattes,F.M., Hainsworth,E.G., Hassan-Walker,A.F., Burroughs,A.K., Sweny,P., Griffiths,P.D., and Emery,V.C. (2005). Kinetics of cytomegalovirus load decrease in solid-organ transplant recipients after preemptive therapy with valganciclovir. J. Infect. Dis. *191*, 89-92.

Mattes,F.M., McLaughlin,J.E., Emery,V.C., Clark,D.A., and Griffiths,P.D. (2000). Histopathological detection of owl's eye inclusions is still specific for

cytomegalovirus in the era of human herpesviruses 6 and 7. J. Clin Pathol. *53*, 612-614.

Mattsson,J., Uzunel,M., Tammik,L., Aschan,J., and Ringden,O. (2001). Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. Leukemia *15*, 1976-1985.

McGavran,M.H. and Smith,M.G. (1965). Ultrastructural, cytochemical, and microchemical observaton of cytomegalovirus (salivary gland virus) infections of human cells in tissue culture. Exp Mol. Pathol. *76*, 1-10.

McGeoch,D.J., Cook,S., Dolan,A., Jamieson,F.E., and Telford,E.A. (1995). Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J Mol. Biol. *247*, 443-458.

McGlave,P.B., Beatty,P., Ash,R., and Hows,J.M. (1990). Therapy for chronic myelogenous leukemia with unrelated donor bone marrow transplantation: results in 102 cases. Blood *75*, 1728-1732.

McSweeney,P.A., Niederwieser,D., Shizuru,J.A., Sandmaier,B.M., Molina,A.J., Maloney,D.G., Chauncey,T.R., Gooley,T.A., Hegenbart,U., Nash,R.A., Radich,J., Wagner,J.L., Minor,S., Appelbaum,F.R., Bensinger,W.I., Bryant,E., Flowers,M.E., Georges,G.E., Grumet,F.C., Kiem,H.P., Torok-Storb,B., Yu,C., Blume,K.G., and Storb,R.F. (2001). Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. Blood *97*, 3390-3400.

McVoy,M.A. and Adler,S.P. (1994). Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. J Virol *68*, 1040-1051.

Meijer,E., Dekker,A.W., Rozenberg-Arska,M., Weersink,A.J., and Verdonck,L.F. (2002). Influence of cytomegalovirus seropositivity on outcome after T cell-depleted bone marrow transplantation: contrasting results between recipients of grafts from related and unrelated donors. Clin Infect. Dis. *35*, 703-712.

Melnick,J.L., Adam,E., and Debakey,M.E. (1993). Cytomegalovirus and atherosclerosis. Eur. Heart J. *14 Suppl K*, 30-38.

Mendelson,M., Monard,S., Sissons,P., and Sinclair,J. (1996). Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. J Gen. Virol *77 (Pt 12)*, 3099-3102.

Mendez,J., Espy,M., Smith,T.F., Wilson,J., Wiesner,R., and Paya,C.V. (1998). Clinical significance of viral load in the diagnosis of cytomegalovirus disease after liver transplantation. Transplantation *65*, 1477-1481.

Merup,M., Lazarevic,V., Nahi,H., Andreasson,B., Malm,C., Nilsson,L., Brune,M., LeBlanc,K., Kutti,J., and Birgegard,G. (2006). Different outcome of

allogeneic transplantation in myelofibrosis using conventional or reduced-intensity conditioning regimens. Br. J. Haematol. *135*, 367-373.

Meyer,A.L., Trollmo,C., Crawford,F., Marrack,P., Steere,A.C., Huber,B.T., Kappler,J., and Hafler,D.A. (2000). Direct enumeration of Borrelia-reactive CD4 T cells ex vivo by using MHC class II tetramers. Proc. Natl. Acad. Sci. U. S. A *97*, 11433-11438.

Meyers,J.D., Flournoy,N., and Thomas,E.D. (1986). Risk factors for cytomegalovirus infection after human marrow transplantation. J. Infect. Dis. *153*, 478-488.

Meyers,J.D., Ljungman,P., and Fisher,L.D. (1990). Cytomegalovirus excretion as a predictor of cytomegalovirus disease after marrow transplantation: importance of cytomegalovirus viremia. J. Infect. Dis. *162*, 373-380.

Meyers,J.D., Reed,E.C., Shepp,D.H., Thornquist,M., Dandliker,P.S., Vicary,C.A., Flournoy,N., Kirk,L.E., Kersey,J.H., Thomas,E.D., and . (1988). Acyclovir for prevention of cytomegalovirus infection and disease after allogeneic marrow transplantation. N. Engl. J. Med *318*, 70-75.

Michallet,M., Archimbaud,E., Bandini,G., Rowlings,P.A., Deeg,H.J., Gahrton,G., Montserrat,E., Rozman,C., Gratwohl,A., and Gale,R.P. (1996). HLA-identical sibling bone marrow transplantation in younger patients with chronic lymphocytic leukemia. European Group for Blood and Marrow Transplantation and the International Bone Marrow Transplant Registry. Ann. Intern. Med. *124*, 311-315.

Miller,W., Flynn,P., McCullough,J., Balfour,H.H., Jr., Goldman,A., Haake,R., McGlave,P., Ramsay,N., and Kersey,J. (1986). Cytomegalovirus infection after bone marrow transplantation: an association with acute graft-v-host disease. Blood *67*, 1162-1167.

Miller,W.J., McCullough,J., Balfour,H.H., Jr., Haake,R.J., Ramsay,N.K., Goldman,A., Bowman,R., and Kersey,J. (1991). Prevention of cytomegalovirus infection following bone marrow transplantation: a randomized trial of blood product screening. Bone Marrow Transplant. *7*, 227-234.

Mocarski,E.S., Jr. (2002). Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. Trends Microbiol. *10*, 332-339.

Mohty,M., Bilger,K., Jourdan,E., Kuentz,M., Michallet,M., Bourhis,J.H., Milpied,N., Sutton,L., Jouet,J.P., Attal,M., Bordigoni,P., Cahn,J.Y., Sadoun,A., Ifrah,N., Guyotat,D., Faucher,C., Fegueux,N., Reiffers,J., Maraninchi,D., and Blaise,D. (2003a). Higher doses of CD34+ peripheral blood stem cells are associated with increased mortality from chronic graft-versus-host disease after allogeneic HLA-identical sibling transplantation. Leukemia *17*, 869-875.

Mohty,M., Faucher,C., Vey,N., Stoppa,A.M., Viret,F., Chabbert,I., Chabannon,C., Bouabdallah,R., Ladaique,P., Collet,L., Zandotti,C., Maraninchi,D., and Blaise,D. (2000). High rate of secondary viral and bacterial

infections in patients undergoing allogeneic bone marrow mini-transplantation. Bone Marrow Transplant. *26*, 251-255.

Mohty,M., Jacot,W., Faucher,C., Bay,J.O., Zandotti,C., Collet,L., Choufi,B., Bilger,K., Tournilhac,O., Vey,N., Stoppa,A.M., Coso,D., Gastaut,J.A., Viens,P., Maraninchi,D., Olive,D., and Blaise,D. (2003b). Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin-based reduced intensity preparative regimen. Leukemia *17*, 2168-2177.

Mori,T., Aisa,Y., Shimizu,T., Nakazato,T., Yamazaki,R., Ikeda,Y., and Okamoto,S. (2006). Prevention of cytomegalovirus infection by valaciclovir after allogeneic bone marrow transplantation from an unrelated donor. Int. J. Hematol. *83*, 266-270.

Morris,E., Thomson,K., Craddock,C., Mahendra,P., Milligan,D., Cook,G., Smith,G.M., Parker,A., Schey,S., Chopra,R., Hatton,C., Tighe,J., Hunter,A., Peggs,K., Linch,D., Goldstone,A., and Mackinnon,S. (2004). Outcomes after alemtuzumab-containing reduced-intensity allogeneic transplantation regimen for relapsed and refractory non-Hodgkin lymphoma. Blood *104*, 3865-3871.

Morris,E.C., Rebello,P., Thomson,K.J., Peggs,K.S., Kyriakou,C., Goldstone,A.H., Mackinnon,S., and Hale,G. (2003). Pharmacokinetics of alemtuzumab used for in vivo and in vitro T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. Blood *102*, 404-406.

Murphy,E., Rigoutsos,I., Shibuya,T., and Shenk,T.E. (2003a). Reevaluation of human cytomegalovirus coding potential. Proc. Natl. Acad. Sci. U. S. A *100*, 13585-13590.

Murphy,E., Yu,D., Grimwood,J., Schmutz,J., Dickson,M., Jarvis,M.A., Hahn,G., Nelson,J.A., Myers,R.M., and Shenk,T.E. (2003b). Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc. Natl. Acad. Sci. U. S. A *100*, 14976-14981.

Nachbaur,D., Clausen,J., and Kircher,B. (2006). Donor cytomegalovirus seropositivity and the risk of leukemic relapse after reduced-intensity transplants. Eur. J. Haematol. *76*, 414-419.

Narimatsu,H., Morishita,Y., Saito,S., Shimada,K., Ozeki,K., Kohno,A., and Kato,Y. (2004). Conditioning regimen of melphalan, fludarabine and total body irradiation in unmanipulated HLA haploidentical stem cell transplantation based on feto-maternal tolerance. Intern Med *43*, 1063-1067.

Nichols,W.G., Corey,L., Gooley,T., Davis,C., and Boeckh,M. (2002). High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J. Infect. Dis. *185*, 273-282.

Nicholson,V.A., Whimbey,E., Champlin,R., bi-Said,D., Przepiorka,D., Tarrand,J., Chan,K., Bodey,G.P., and Goodrich,J.M. (1997). Comparison of cytomegalovirus antigenemia and shell vial culture in allogeneic marrow transplantation recipients receiving ganciclovir prophylaxis. Bone Marrow Transplant. *19*, 37-41.

Nielsen,M., Lundegaard,C., Worning,P., Hvid,C.S., Lamberth,K., Buus,S., Brunak,S., and Lund,O. (2004). Improved prediction of MHC class I and class II epitopes using a novel Gibbs sampling approach. Bioinformatics. *20*, 1388-1397.

Nolte,F.S., Emmens,R.K., Thurmond,C., Mitchell,P.S., Pascuzzi,C., Devine,S.M., Saral,R., and Wingard,J.R. (1995). Early detection of human cytomegalovirus viremia in bone marrow transplant recipients by DNA amplification. J. Clin Microbiol. *33*, 1263-1266.

Novitzky,N., Thomas,V., Hale,G., and Waldmann,H. (2004). Campath-1 Abs 'in the bag' for hematological malignancies: the Cape Town experience. Cytotherapy. *6*, 172-181.

Novotny,J., Rigoutsos,I., Coleman,D., and Shenk,T. (2001). In silico structural and functional analysis of the human cytomegalovirus (HHV5) genome. J Mol. Biol. *310*, 1151-1166.

Obama,K., Utsunomiya,A., Takatsuka,Y., and Takemoto,Y. (2004). Reduced-intensity non-T-cell depleted HLA-haploidentical stem cell transplantation for older patients based on the concept of feto-maternal tolerance. Bone Marrow Transplant. *34*, 897-899.

Olsson,J., Wikby,A., Johansson,B., Lofgren,S., Nilsson,B.O., and Ferguson,F.G. (2000). Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. Mech. Ageing Dev. *121*, 187-201.

Osgood, E. E., Riddle, M. C., and Mathews, T. J. Ann Intern Med 13, 357-367. 1939.
Ref Type: Generic

Ouyang,Q., Wagner,W.M., Wikby,A., Walter,S., Aubert,G., Dodi,A.I., Travers,P., and Pawelec,G. (2003). Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. J. Clin Immunol. *23*, 247-257.

Pamphilon,D.H., Rider,J.R., Barbara,J.A., and Williamson,L.M. (1999). Prevention of transfusion-transmitted cytomegalovirus infection. Transfus. Med *9*, 115-123.

Pass,R.F. (1985). Epidemiology and transmission of cytomegalovirus. J Infect. Dis *152*, 243-248.

Pass,R.F., Duliege,A.M., Boppana,S., Sekulovich,R., Percell,S., Britt,W., and Burke,R.L. (1999). A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. J. Infect. Dis. *180*, 970-975.

Pass,R.F., Hutto,C., Lyon,M.D., and Cloud,G. (1990). Increased rate of cytomegalovirus infection among day care center workers. Pediatr. Infect. Dis. J. *9*, 465-470.

Pass,R.F., Hutto,S.C., Reynolds,D.W., and Polhill,R.B. (1984). Increased frequency of cytomegalovirus infection in children in group day care. Pediatrics *74*, 121-126.

Passweg,J.R., Perez,W.S., Eapen,M., Camitta,B.M., Gluckman,E., Hinterberger,W., Hows,J.M., Marsh,J.C., Pasquini,R., Schrezenmeier,H., Socie,G., Zhang,M.J., and Bredeson,C. (2006). Bone marrow transplants from mismatched related and unrelated donors for severe aplastic anemia. Bone Marrow Transplant. *37*, 641-649.

Paston,S.J., Dodi,I.A., and Madrigal,J.A. (2004). Progress made towards the development of a CMV peptide vaccine. Hum. Immunol. *65*, 544-549.

Patriarca,F., Skert,C., Sperotto,A., Zaja,F., Falleti,E., Mestroni,R., Kikic,F., Calistri,E., Fili,C., Geromin,A., Cerno,M., and Fanin,R. (2006). The development of autoantibodies after allogeneic stem cell transplantation is related with chronic graft-vs-host disease and immune recovery 6. Exp Hematol. *34*, 389-396.

Pavletic,Z.S., Arrowsmith,E.R., Bierman,P.J., Goodman,S.A., Vose,J.M., Tarantolo,S.R., Stein,R.S., Bociek,G., Greer,J.P., Wu,C.D., Kollath,J.P., Weisenburger,D.D., Kessinger,A., Wolff,S.N., Armitage,J.O., and Bishop,M.R. (2000). Outcome of allogeneic stem cell transplantation for B cell chronic lymphocytic leukemia. Bone Marrow Transplant. *25*, 717-722.

Pawson,R., Potter,M.N., Theocharous,P., Lawler,M., Garg,M., Yin,J.A., Rezvani,K., Craddock,C., Rassam,S., and Prentice,H.G. (2001). Treatment of relapse after allogeneic bone marrow transplantation with reduced intensity conditioning (FLAG +/- Ida) and second allogeneic stem cell transplant. Br. J Haematol. *115*, 622-629.

Payne,R., Tripp,M.J., Weigle,J., Bodmer,W., and Bodmer,J. (1964). A new leukocyte isoantigen system in man. Cold Spring Harb. Symp. Quant. Biol. *29*, 285-295.

Peggs,K., Verfuerth,S., and Mackinnon,S. (2001). Induction of cytomegalovirus (CMV)-specific T-cell responses using dendritic cells pulsed with CMV antigen: a novel culture system free of live CMV virions. Blood *97*, 994-1000.

Peggs,K.S., Hunter,A., Chopra,R., Parker,A., Mahendra,P., Milligan,D., Craddock,C., Pettengell,R., Dogan,A., Thomson,K.J., Morris,E.C., Hale,G., Waldmann,H., Goldstone,A.H., Linch,D.C., and Mackinnon,S. (2005). Clinical evidence of a graft-versus-Hodgkin's-lymphoma effect after reduced-intensity allogeneic transplantation. Lancet *365*, 1934-1941.

Peggs,K.S., Mackinnon,S., Williams,C.D., D'Sa,S., Thuraisundaram,D., Kyriakou,C., Morris,E.C., Hale,G., Waldmann,H., Linch,D.C., Goldstone,A.H.,

and Yong,K. (2003a). Reduced-intensity transplantation with in vivo T-cell depletion and adjuvant dose-escalating donor lymphocyte infusions for chemotherapy-sensitive myeloma: limited efficacy of graft-versus-tumor activity. Biol. Blood Marrow Transplant. *9*, 257-265.

Peggs,K.S., Preiser,W., Kottaridis,P.D., McKeag,N., Brink,N.S., Tedder,R.S., Goldstone,A.H., Linch,D.C., and Mackinnon,S. (2000). Extended routine polymerase chain reaction surveillance and pre-emptive antiviral therapy for cytomegalovirus after allogeneic transplantation. Br. J Haematol. *111*, 782-790.

Peggs,K.S., Verfuerth,S., Pizzey,A., Khan,N., Moss,P., Goldstone,A.H., Yong,K., and Mackinnon,S. (2003b). Reconstitution of T-cell repertoire after autologous stem cell transplantation: influence of CD34 selection and cytomegalovirus infection. Biol. Blood Marrow Transplant. *9*, 198-205.

Penfold,M.E., Dairaghi,D.J., Duke,G.M., Saederup,N., Mocarski,E.S., Kemble,G.W., and Schall,T.J. (1999). Cytomegalovirus encodes a potent alpha chemokine. Proc. Natl. Acad. Sci. U. S. A *96*, 9839-9844.

Petersdorf,E.W., Longton,G.M., Anasetti,C., Martin,P.J., Mickelson,E.M., Smith,A.G., and Hansen,J.A. (1995). The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. Blood *86*, 1606-1613.

Petersen,S.L., Ryder,L.P., Bjork,P., Madsen,H.O., Heilmann,C., Jacobsen,N., Sengelov,H., and Vindelov,L.L. (2003). A comparison of T-, B- and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors
27. Bone Marrow Transplant. *32*, 65-72.

Philip,T., Armitage,J.O., Spitzer,G., Chauvin,F., Jagannath,S., Cahn,J.Y., Colombat,P., Goldstone,A.H., Gorin,N.C., Flesh,M., and . (1987). High-dose therapy and autologous bone marrow transplantation after failure of conventional chemotherapy in adults with intermediate-grade or high-grade non-Hodgkin's lymphoma. N. Engl. J. Med. *316*, 1493-1498.

Phillips,J., Drumm,A., Harrison,P., Bird,P., Bhamra,K., Berrie,E., and Hale,G. (2001). Manufacture and quality control of CAMPATH-1 antibodies for clinical trials. Cytotherapy. *3*, 233-242.

Pignatelli,S., Dal,M.P., Rossini,G., and Landini,M.P. (2004). Genetic polymorphisms among human cytomegalovirus (HCMV) wild-type strains
6. Rev. Med Virol. *14*, 383-410.

Pitcher,C.J., Quittner,C., Peterson,D.M., Connors,M., Koup,R.A., Maino,V.C., and Picker,L.J. (1999). HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. *5*, 518-525.

Plotkin,S.A., Farquhar,J., and Horberger,E. (1976). Clinical trials of immunization with the Towne 125 strain of human cytomegalovirus. J. Infect. Dis. *134*, 470-475.

Plotkin,S.A., Smiley,M.L., Friedman,H.M., Starr,S.E., Fleisher,G.R., Wlodaver,C., Dafoe,D.C., Friedman,A.D., Grossman,R.A., and Barker,C.F. (1984). Towne-vaccine-induced prevention of cytomegalovirus disease after renal transplants. Lancet *1*, 528-530.

Plotkin,S.A., Starr,S.E., Friedman,H.M., Brayman,K., Harris,S., Jackson,S., Tustin,N.B., Grossman,R., Dafoe,D., and Barker,C. (1991). Effect of Towne live virus vaccine on cytomegalovirus disease after renal transplant. A controlled trial 16. Ann. Intern. Med *114*, 525-531.

Polis,M.A., Spooner,K.M., Baird,B.F., Manischewitz,J.F., Jaffe,H.S., Fisher,P.E., Falloon,J., Davey,R.T., Jr., Kovacs,J.A., Walker,R.E., and . (1995). Anticytomegaloviral activity and safety of cidofovir in patients with human immunodeficiency virus infection and cytomegalovirus viruria. Antimicrob. Agents Chemother. *39*, 882-886.

Poncet,D., Larochette,N., Pauleau,A.L., Boya,P., Jalil,A.A., Cartron,P.F., Vallette,F., Schnebelen,C., Bartle,L.M., Skaletskaya,A., Boutolleau,D., Martinou,J.C., Goldmacher,V.S., Kroemer,G., and Zamzami,N. (2004). An anti-apoptotic viral protein that recruits Bax to mitochondria. J Biol. Chem. *279*, 22605-22614.

Porter,D.L., Collins,R.H., Jr., Hardy,C., Kernan,N.A., Drobyski,W.R., Giralt,S., Flowers,M.E., Casper,J., Leahey,A., Parker,P., Mick,R., Bate-Boyle,B., King,R., and Antin,J.H. (2000). Treatment of relapsed leukemia after unrelated donor marrow transplantation with unrelated donor leukocyte infusions. Blood *95*, 1214-1221.

Powles,R., Mehta,J., Kulkarni,S., Treleaven,J., Millar,B., Marsden,J., Shepherd,V., Rowland,A., Sirohi,B., Tait,D., Horton,C., Long,S., and Singhal,S. (2000). Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. Lancet *355*, 1231-1237.

Prentice,H.G., Blacklock,H.A., Janossy,G., Bradstock,K.F., Skeggs,D., Goldstein,G., and Hoffbrand,A.V. (1982). Use of anti-T-cell monoclonal antibody OKT3 to prevent acute graft-versus-host disease in allogeneic bone-marrow transplantation for acute leukaemia. Lancet *1*, 700-703.

Prentice,H.G., Blacklock,H.A., Janossy,G., Gilmore,M.J., Price-Jones,L., Tidman,N., Trejdosiewicz,L.K., Skeggs,D.B., Panjwani,D., Ball,S., and . (1984). Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients. Lancet *1*, 472-476.

Prentice,H.G., Gluckman,E., Powles,R.L., Ljungman,P., Milpied,N., Fernandez Ranada,J.M., Mandelli,F., Kho,P., Kennedy,L., and Bell,A.R. (1994). Impact of long-term acyclovir on cytomegalovirus infection and survival after allogeneic

bone marrow transplantation. European Acyclovir for CMV Prophylaxis Study Group. Lancet *343*, 749-753.

Prentice,H.G., Gluckman,E., Powles,R.L., Ljungman,P., Milpied,N.J., Camara,R., Mandelli,F., Kho,P., Kennedy,L., and Bell,A.R. (1997). Long-term survival in allogeneic bone marrow transplant recipients following acyclovir prophylaxis for CMV infection. The European Acyclovir for CMV Prophylaxis Study Group. Bone Marrow Transplant. *19*, 129-133.

Prichard,M.N., Penfold,M.E., Duke,G.M., Spaete,R.R., and Kemble,G.W. (2001). A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. Rev. Med Virol. *11*, 191-200.

Pui,C.H. and Evans,W.E. (2006). Treatment of acute lymphoblastic leukemia. N. Engl. J. Med. *354*, 166-178.

Quinnan,G.V., Jr., Delery,M., Rook,A.H., Frederick,W.R., Epstein,J.S., Manischewitz,J.F., Jackson,L., Ramsey,K.M., Mittal,K., Plotkin,S.A., and . (1984). Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. Ann Intern Med *101*, 478-483.

Quinnan,G.V., Jr., Kirmani,N., Esber,E., Saral,R., Manischewitz,J.F., Rogers,J.L., Rook,A.H., Santos,G.W., and Burns,W.H. (1981). HLA-restricted cytotoxic T lymphocyte and nonthymic cytotoxic lymphocyte responses to cytomegalovirus infection of bone marrow transplant recipients. J. Immunol. *126*, 2036-2041.

Raeiszadeh,M., Kopycinski,J., Paston,S.J., Diss,T., Lowdell,M., Hardy,G.A., Hislop,A.D., Workman,S., Dodi,A., Emery,V., and Webster,A.D. (2006). The T cell response to persistent herpes virus infections in common variable immunodeficiency. Clin Exp. Immunol. *146*, 234-242.

Rammensee,H., Bachmann,J., Emmerich,N.P., Bachor,O.A., and Stevanovic,S. (1999). SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics *50*, 213-219.

Rammensee,H.G. (1995). Chemistry of peptides associated with MHC class I and class II molecules
5. Curr. Opin. Immunol. *7*, 85-96.

Rauser,G., Einsele,H., Sinzger,C., Wernet,D., Kuntz,G., Assenmacher,M., Campbell,J.D., and Topp,M.S. (2004). Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. Blood *103*, 3565-3572.

rav-Boger,R., Foster,C.B., Zong,J.C., and Pass,R.F. (2006). Human cytomegalovirus-encoded alpha -chemokines exhibit high sequence variability in congenitally infected newborns. J Infect. Dis *193*, 788-791.

Rebello,P., Cwynarski,K., Varughese,M., Eades,A., Apperley,J.F., and Hale,G. (2001). Pharmacokinetics of CAMPATH-1H in BMT patients. Cytotherapy. *3*, 261-267.

Reche,P.A. and Reinherz,E.L. (2005). PEPVAC: a web server for multi-epitope vaccine development based on the prediction of supertypic MHC ligands. Nucleic Acids Res. *33*, W138-W142.

Reddehase,M.J. (2002). Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat. Rev. Immunol. *2*, 831-844.

Reddehase,M.J., Simon,C.O., Podlech,J., and Holtappels,R. (2004). Stalemating a clever opportunist: lessons from murine cytomegalovirus. Hum. Immunol. *65*, 446-455.

Reed,E.C., Bowden,R.A., Dandliker,P.S., Lilleby,K.E., and Meyers,J.D. (1988). Treatment of cytomegalovirus pneumonia with ganciclovir and intravenous cytomegalovirus immunoglobulin in patients with bone marrow transplants. Ann Intern Med *109*, 783-788.

Reed,E.C., Wolford,J.L., Kopecky,K.J., Lilleby,K.E., Dandliker,P.S., Todaro,J.L., McDonald,G.B., and Meyers,J.D. (1990). Ganciclovir for the treatment of cytomegalovirus gastroenteritis in bone marrow transplant patients. A randomized, placebo-controlled trial. Ann Intern Med *112*, 505-510.

Regoes,R.R., Bowen,E.F., Cope,A.V., Gor,D., Hassan-Walker,A.F., Prentice,H.G., Johnson,M.A., Sweny,P., Burroughs,A.K., Griffiths,P.D., Bonhoeffer,S., and Emery,V.C. (2006). Modelling cytomegalovirus replication patterns in the human host: factors important for pathogenesis. Proc. Biol. Sci. *273*, 1961-1967.

Reinke,P., Prosch,S., Kern,F., and Volk,H.D. (1999). Mechanisms of human cytomegalovirus (HCMV) (re)activation and its impact on organ transplant patients. Transpl. Infect. Dis. *1*, 157-164.

Reusser,P., Cathomas,G., Attenhofer,R., Tamm,M., and Thiel,G. (1999). Cytomegalovirus (CMV)-specific T cell immunity after renal transplantation mediates protection from CMV disease by limiting the systemic virus load. J. Infect. Dis. *180*, 247-253.

Reusser,P., Cordonnier,C., Einsele,H., Engelhard,D., Link,D., Locasciulli,A., and Ljungman,P. (1996). European survey of herpesvirus resistance to antiviral drugs in bone marrow transplant recipients. Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation (EBMT). Bone Marrow Transplant. *17*, 813-817.

Reusser,P., Einsele,H., Lee,J., Volin,L., Rovira,M., Engelhard,D., Finke,J., Cordonnier,C., Link,H., and Ljungman,P. (2002). Randomized multicenter trial of foscarnet versus ganciclovir for preemptive therapy of cytomegalovirus infection after allogeneic stem cell transplantation. Blood *99*, 1159-1164.

Reusser,P., Riddell,S.R., Meyers,J.D., and Greenberg,P.D. (1991). Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood *78*, 1373-1380.

Revello,M.G., Percivalle,E., Di,M.A., Morini,F., and Gerna,G. (1992). Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viraemic patients. J. Gen. Virol. *73 (Pt 2)*, 437-442.

Riddell,S.R. (1995). Pathogenesis of cytomegalovirus pneumonia in immunocompromised hosts. Semin. Respir. Infect. *10*, 199-208.

Riddell,S.R., Watanabe,K.S., Goodrich,J.M., Li,C.R., Agha,M.E., and Greenberg,P.D. (1992). Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science *257*, 238-241.

Ridge,J.P., Di,R.F., and Matzinger,P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Rigoutsos,I., Novotny,J., Huynh,T., Chin-Bow,S.T., Parida,L., Platt,D., Coleman,D., and Shenk,T. (2003). In silico pattern-based analysis of the human cytomegalovirus genome. J Virol *77*, 4326-4344.

Ringden,O., Pihlstedt,P., Volin,L., Nikoskelainen,J., Lonnqvist,B., Ruutu,P., Ruutu,T., Toivanen,A., and Wahren,B. (1987). Failure to prevent cytomegalovirus infection by cytomegalovirus hyperimmune plasma: a randomized trial by the Nordic Bone Marrow Transplantation Group. Bone Marrow Transplant. *2*, 299-305.

Robinson,S.P., Goldstone,A.H., Mackinnon,S., Carella,A., Russell,N., de Elvira,C.R., Taghipour,G., and Schmitz,N. (2002). Chemoresistant or aggressive lymphoma predicts for a poor outcome following reduced-intensity allogeneic progenitor cell transplantation: an analysis from the Lymphoma Working Party of the European Group for Blood and Bone Marrow Transplantation. Blood *100*, 4310-4316.

Roby,C. and Gibson,W. (1986). Characterization of phosphoproteins and protein kinase activity of virions, noninfectious enveloped particles, and dense bodies of human cytomegalovirus. J Virol *59*, 714-727.

Roizman,B. (1995). Sixth Report of the International Committee on the Taxonomy of Viruses. Arch Virol *140*, 1-586.

Rondelli,D., Barosi,G., Bacigalupo,A., Prchal,J.T., Popat,U., Alessandrino,E.P., Spivak,J.L., Smith,B.D., Klingemann,H.G., Fruchtman,S., and Hoffman,R. (2005). Allogeneic hematopoietic stem-cell transplantation with reduced-intensity conditioning in intermediate- or high-risk patients with myelofibrosis with myeloid metaplasia. Blood *105*, 4115-4119.

Ross,R. (1999). Atherosclerosis--an inflammatory disease. N. Engl. J Med *340*, 115-126.

Roux,E., Dumont-Girard,F., Starobinski,M., Siegrist,C.A., Helg,C., Chapuis,B., and Roosnek,E. (2000). Recovery of immune reactivity after T-cell-depleted

bone marrow transplantation depends on thymic activity
1. Blood *96*, 2299-2303.

Roux,E., Helg,C., Dumont-Girard,F., Chapuis,B., Jeannet,M., and Roosnek,E. (1996). Analysis of T-cell repopulation after allogeneic bone marrow transplantation: significant differences between recipients of T-cell depleted and unmanipulated grafts
2. Blood *87*, 3984-3992.

Rowe,W.P., Hartley,J.W., Waterman,S., Turner,H.C., and Huebner,R.J. (1956). Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. Exp Biol Med *92*, 418-424.

Rubin,R.H. (1990). Impact of cytomegalovirus infection on organ transplant recipients. Rev. Infect. Dis *12 Suppl 7*, S754-S766.

Rubin,R.H. (1997). Cytomegalovirus infection in the liver transplant recipient. Epidemiology, pathogenesis, and clinical management
21. Clin Liver Dis. *1*, 439-52, x.

Russell,N.H., Hunter,A., Rogers,S., Hanley,J., and Anderson,D. (1993). Peripheral blood stem cells as an alternative to marrow for allogeneic transplantation. Lancet *341*, 1482.

Sacre,K., Carcelain,G., Cassoux,N., Fillet,A.M., Costagliola,D., Vittecoq,D., Salmon,D., Amoura,Z., Katlama,C., and Autran,B. (2005). Repertoire, diversity, and differentiation of specific CD8 T cells are associated with immune protection against human cytomegalovirus disease. J. Exp. Med. *201*, 1999-2010.

Saederup,N. and Mocarski,E.S., Jr. (2002). Fatal attraction: cytomegalovirus-encoded chemokine homologs. Curr. Top. Microbiol. Immunol. *269*, 235-256.

Saffert,R.T. and Kalejta,R.F. (2006). Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. J Virol *80*, 3863-3871.

Sageda,S., Nordal,K.P., Hartmann,A., Sund,S., Scott,H., Degre,M., Foss,A., Leivestad,T., Osnes,K., Fauchald,P., and Rollag,H. (2002). The impact of cytomegalovirus infection and disease on rejection episodes in renal allograft recipients. Am J Transplant. *2*, 850-856.

Sallusto,F., Lenig,D., Forster,R., Lipp,M., and Lanzavecchia,A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions
2. Nature *401*, 708-712.

Salomon,J. and Flower,D.R. (2006). Predicting Class II MHC-Peptide binding: a kernel based approach using similarity scores. BMC. Bioinformatics. *7*, 501.

Salomon,N. and Perlman,D.C. (1999). Cytomegalovirus pneumonia
22. Semin. Respir. Infect. *14*, 353-358.

Salzberger,B., Bowden,R.A., Hackman,R.C., Davis,C., and Boeckh,M. (1997). Neutropenia in allogeneic marrow transplant recipients receiving ganciclovir for prevention of cytomegalovirus disease: risk factors and outcome. Blood *90*, 2502-2508.

Santos,G.W., Tutschka,P.J., Brookmeyer,R., Saral,R., Beschorner,W.E., Bias,W.B., Braine,H.G., Burns,W.H., Elfenbein,G.J., Kaizer,H., and . (1983). Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. N. Engl. J. Med *309*, 1347-1353.

Sasazuki,T., Juji,T., Morishima,Y., Kinukawa,N., Kashiwabara,H., Inoko,H., Yoshida,T., Kimura,A., Akaza,T., Kamikawaji,N., Kodera,Y., and Takaku,F. (1998). Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. N. Engl. J. Med *339*, 1177-1185.

Schetelig,J., Thiede,C., Bornhauser,M., Schwerdtfeger,R., Kiehl,M., Beyer,J., Sayer,H.G., Kroger,N., Hensel,M., Scheffold,C., Held,T.K., Hoffken,K., Ho,A.D., Kienast,J., Neubauer,A., Zander,A.R., Fauser,A.A., Ehninger,G., and Siegert,W. (2003). Evidence of a graft-versus-leukemia effect in chronic lymphocytic leukemia after reduced-intensity conditioning and allogeneic stem-cell transplantation: the Cooperative German Transplant Study Group. J. Clin. Oncol. *21*, 2747-2753.

Schleiss,M.R. and Heineman,T.C. (2005). Progress toward an elusive goal: current status of cytomegalovirus vaccines
11. Expert. Rev. Vaccines. *4*, 381-406.

Schleiss,M.R., Stroup,G., Pogorzelski,K., and McGregor,A. (2006). Protection against congenital cytomegalovirus (CMV) disease, conferred by a replication-disabled, bacterial artificial chromosome (BAC)-based DNA vaccine
6. Vaccine *24*, 6175-6186.

Schmid,C., Schleuning,M., Ledderose,G., Tischer,J., and Kolb,H.J. (2005). Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. J. Clin. Oncol. *23*, 5675-5687.

Schmidt,G.M., Horak,D.A., Niland,J.C., Duncan,S.R., Forman,S.J., and Zaia,J.A. (1991). A randomized, controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants; The City of Hope-Stanford-Syntex CMV Study Group. N. Engl. J. Med *324*, 1005-1011.

Schmidt,G.M., Kovacs,A., Zaia,J.A., Horak,D.A., Blume,K.G., Nademanee,A.P., O'Donnell,M.R., Snyder,D.S., and Forman,S.J. (1988). Ganciclovir/immunoglobulin combination therapy for the treatment of human cytomegalovirus-associated interstitial pneumonia in bone marrow allograft recipients. Transplantation *46*, 905-907.

Schmitz,N., Beksac,M., Hasenclever,D., Bacigalupo,A., Ruutu,T., Nagler,A., Gluckman,E., Russell,N., Apperley,J.F., Gorin,N.C., Szer,J., Bradstock,K., Buzyn,A., Clark,P., Borkett,K., and Gratwohl,A. (2002). Transplantation of mobilized peripheral blood cells to HLA-identical siblings with standard-risk leukemia. Blood *100*, 761-767.

Schmolke,S., Drescher,P., Jahn,G., and Plachter,B. (1995a). Nuclear targeting of the tegument protein pp65 (UL83) of human cytomegalovirus: an unusual bipartite nuclear localization signal functions with other portions of the protein to mediate its efficient nuclear transport. J Virol *69*, 1071-1078.

Schmolke,S., Kern,H.F., Drescher,P., Jahn,G., and Plachter,B. (1995b). The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture. J Virol *69*, 5959-5968.

Schoemans,H., Theunissen,K., Maertens,J., Boogaerts,M., Verfaillie,C., and Wagner,J. (2006). Adult umbilical cord blood transplantation: a comprehensive review. Bone Marrow Transplant. *38*, 83-93.

Schoenberger,S.P., Toes,R.E., van,d., V, Offringa,R., and Melief,C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schoppel,K., Kropff,B., Schmidt,C., Vornhagen,R., and Mach,M. (1997). The humoral immune response against human cytomegalovirus is characterized by a delayed synthesis of glycoprotein-specific antibodies. J. Infect. Dis. *175*, 533-544.

Seder,R.A. and Ahmed,R. (2003). Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat. Immunol. *4*, 835-842.

Sester,M., Sester,U., Gartner,B., Heine,G., Girndt,M., Mueller-Lantzsch,N., Meyerhans,A., and Kohler,H. (2001). Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. Transplantation *71*, 1287-1294.

Sester,M., Sester,U., Gartner,B., Kubuschok,B., Girndt,M., Meyerhans,A., and Kohler,H. (2002). Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus. J. Virol. *76*, 3748-3755.

Shedlock,D.J. and Shen,H. (2003). Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science *300*, 337-339.

Shellam,G.R., Allan,J.E., Papadimitriou,J.M., and Bancroft,G.J. (1981). Increased susceptibility to cytomegalovirus infection in beige mutant mice 3. Proc. Natl. Acad. Sci. U. S. A *78*, 5104-5108.

Shepp,D.H., Moses,J.E., and Kaplan,M.H. (1996). Seroepidemiology of cytomegalovirus in patients with advanced HIV disease: influence on disease expression and survival 3. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. *11*, 460-468.

Shulman,H.M., Sullivan,K.M., Weiden,P.L., McDonald,G.B., Striker,G.E., Sale,G.E., Hackman,R., Tsoi,M.S., Storb,R., and Thomas,E.D. (1980). Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients
1. Am. J. Med *69*, 204-217.

Simmen,K.A., Singh,J., Luukkonen,B.G., Lopper,M., Bittner,A., Miller,N.E., Jackson,M.R., Compton,T., and Fruh,K. (2001). Global modulation of cellular transcription by human cytomegalovirus is initiated by viral glycoprotein B. Proc. Natl. Acad. Sci. U. S. A *98*, 7140-7145.

Sinclair,E., Black,D., Epling,C.L., Carvidi,A., Josefowicz,S.Z., Bredt,B.M., and Jacobson,M.A. (2004). CMV antigen-specific CD4+ and CD8+ T cell IFNgamma expression and proliferation responses in healthy CMV-seropositive individuals. Viral Immunol. *17*, 445-454.

Sinclair,J. and Sissons,P. (2006). Latency and reactivation of human cytomegalovirus. J Gen. Virol *87*, 1763-1779.

Singhal,S., Shaw,J.C., Ainsworth,J., Hathaway,M., Gillespie,G.M., Paris,H., Ward,K., Pillay,D., Moss,P.A., and Mutimer,D.J. (2000). Direct visualization and quantitation of cytomegalovirus-specific CD8+ cytotoxic T-lymphocytes in liver transplant patients. Transplantation *69*, 2251-2259.

Sinzger,C., Bissinger,A.L., Viebahn,R., Oettle,H., Radke,C., Schmidt,C.A., and Jahn,G. (1999a). Hepatocytes are permissive for human cytomegalovirus infection in human liver cell culture and In vivo. J Infect. Dis *180*, 976-986.

Sinzger,C., Schmidt,K., Knapp,J., Kahl,M., Beck,R., Waldman,J., Hebart,H., Einsele,H., and Jahn,G. (1999b). Modification of human cytomegalovirus tropism through propagation in vitro is associated with changes in the viral genome. J Gen. Virol *80 (Pt 11)*, 2867-2877.

Skaletskaya,A., Bartle,L.M., Chittenden,T., McCormick,A.L., Mocarski,E.S., and Goldmacher,V.S. (2001). A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc. Natl. Acad. Sci. U. S. A *98*, 7829-7834.

Slavin,S., Nagler,A., Naparstek,E., Kapelushnik,Y., Aker,M., Cividalli,G., Varadi,G., Kirschbaum,M., Ackerstein,A., Samuel,S., Amar,A., Brautbar,C., Ben-Tal,O., Eldor,A., and Or,R. (1998). Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood *91*, 756-763.

Smith,I.L., Cherrington,J.M., Jiles,R.E., Fuller,M.D., Freeman,W.R., and Spector,S.A. (1997). High-level resistance of cytomegalovirus to ganciclovir is associated with alterations in both the UL97 and DNA polymerase genes. J. Infect. Dis. *176*, 69-77.

Smith,J.D. (1980). An additional role for the outer nuclear membrane in the morphogenesis of herpes simplex virus. Intervirology *13*, 312-316.

Smith,M.G. (1956). Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease. Proc. Soc. Exp Biol Med *92*, 424-430.

Snydman,D.R. and Falagas,M.E. (1996). Prevention of cytomegalovirus disease in transplant recipients. Lancet *347*, 268-269.

Snydman,D.R., Werner,B.G., Heinze-Lacey,B., Berardi,V.P., Tilney,N.L., Kirkman,R.L., Milford,E.L., Cho,S.I., Bush,H.L., Jr., Levey,A.S., and . (1987). Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. N. Engl. J. Med *317*, 1049-1054.

Soderberg-Naucler,C., Fish,K.N., and Nelson,J.A. (1997). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell *91*, 119-126.

Sokos,D.R., Berger,M., and Lazarus,H.M. (2002). Intravenous immunoglobulin: appropriate indications and uses in hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. *8*, 117-130.

Solache,A., Morgan,C.L., Dodi,A.I., Morte,C., Scott,I., Baboonian,C., Zal,B., Goldman,J., Grundy,J.E., and Madrigal,J.A. (1999). Identification of three HLA-A*0201-restricted cytotoxic T cell epitopes in the cytomegalovirus protein pp65 that are conserved between eight strains of the virus. J. Immunol. *163*, 5512-5518.

Spaete,R.R., Gehrz,R.C., and Landini,M.P. (1994). Human cytomegalovirus structural proteins. J Gen. Virol *75 (Pt 12)*, 3287-3308.

Spector,D.H. (1996). Activation and regulation of human cytomegalovirus early genes. Intervirology *39*, 361-377.

Spector,S.A., Wong,R., Hsia,K., Pilcher,M., and Stempien,M.J. (1998). Plasma cytomegalovirus (CMV) DNA load predicts CMV disease and survival in AIDS patients. J. Clin Invest *101*, 497-502.

Speir,E., Modali,R., Huang,E.S., Leon,M.B., Shawl,F., Finkel,T., and Epstein,S.E. (1994). Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science *265*, 391-394.

Spencer,J.V., Lockridge,K.M., Barry,P.A., Lin,G., Tsang,M., Penfold,M.E., and Schall,T.J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. J Virol *76*, 1285-1292.

Stamminger,T., Gstaiger,M., Weinzierl,K., Lorz,K., Winkler,M., and Schaffner,W. (2002). Open reading frame UL26 of human cytomegalovirus encodes a novel tegument protein that contains a strong transcriptional activation domain. J Virol *76*, 4836-4847.

Stenberg,R.M. (1996). The human cytomegalovirus major immediate-early gene. Intervirology *39*, 343-349.

Stinski,M.F. (1978). Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. *26*, 686-701.

Storb,R., Epstein,R.B., Graham,T.C., and THOMAS,E.D. (1970). Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. Transplantation *9*, 240-246.

Storb,R., Etzioni,R., Anasetti,C., Appelbaum,F.R., Buckner,C.D., Bensinger,W., Bryant,E., Clift,R., Deeg,H.J., Doney,K., and . (1994). Cyclophosphamide combined with antithymocyte globulin in preparation for allogeneic marrow transplants in patients with aplastic anemia. Blood *84*, 941-949.

Storb,R., Prentice,R.L., Buckner,C.D., Clift,R.A., Appelbaum,F., Deeg,J., Doney,K., Hansen,J.A., Mason,M., Sanders,J.E., Singer,J., Sullivan,K.M., Witherspoon,R.P., and Thomas,E.D. (1983). Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N. Engl. J. Med *308*, 302-307.

Storb,R., Prentice,R.L., and THOMAS,E.D. (1977). Treatment of aplastic anemia by marrow transplantation from HLA identical siblings. Prognostic factors associated with graft versus host disease and survival. J. Clin Invest *59*, 625-632.

Storb,R., Thomas,E.D., Buckner,C.D., Clift,R.A., Johnson,F.L., Fefer,A., Glucksberg,H., Giblett,E.R., Lerner,K.G., and Neiman,P. (1974). Allogeneic marrow grafting for treatment of aplastic anemia. Blood *43*, 157-180.

Storek,J., Witherspoon,R.P., and Storb,R. (1995). T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. Bone Marrow Transplant. *16*, 413-425.

Story,C.M., Furman,M.H., and Ploegh,H.L. (1999). The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products. Proc. Natl. Acad. Sci. U. S. A *96*, 8516-8521.

Su,H.C., Cousens,L.P., Fast,L.D., Slifka,M.K., Bungiro,R.D., Ahmed,R., and Biron,C.A. (1998). CD4+ and CD8+ T cell interactions in IFN-gamma and IL-4 responses to viral infections: requirements for IL-2. J. Immunol. *160*, 5007-5017.

Suciu,S., Mandelli,F., De,W.T., Zittoun,R., Gallo,E., Labar,B., De,R.G., Belhabri,A., Giustolisi,R., Delarue,R., Liso,V., Mirto,S., Leone,G., Bourhis,J.H., Fioritoni,G., Jehn,U., Amadori,S., Fazi,P., Hagemeijer,A., and Willemze,R. (2003). Allogeneic compared with autologous stem cell transplantation in the treatment of patients younger than 46 years with acute myeloid leukemia (AML) in first complete remission (CR1): an intention-to-treat analysis of the EORTC/GIMEMAAML-10 trial. Blood *102*, 1232-1240.

Sullivan,K.M., Storek,J., Kopecky,K.J., Jocom,J., Longton,G., Flowers,M., Siadak,M., Nims,J., Witherspoon,R.P., Anasetti,C., Appelbaum,F.R., Bowden,R.A., Buckner,C.D., Crawford,S.W., Deeg,H.J., Hansen,J.A., McDonald,G.B., Sanders,J.E., and Storb,R. (1996). A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs.-host disease after marrow transplantation: clinical outcome and effect on subsequent immune recovery. Biol. Blood Marrow Transplant. *2*, 44-53.

Sullivan,V., Biron,K.K., Talarico,C., Stanat,S.C., Davis,M., Pozzi,L.M., and Coen,D.M. (1993). A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to ganciclovir and phosphonylmethoxyalkyl derivatives. Antimicrob. Agents Chemother. *37*, 19-25.

Sun,J.C. and Bevan,M.J. (2003). Defective CD8 T cell memory following acute infection without CD4 T cell help. Science *300*, 339-342.

Sylwester,A.W., Mitchell,B.L., Edgar,J.B., Taormina,C., Pelte,C., Ruchti,F., Sleath,P.R., Grabstein,K.H., Hosken,N.A., Kern,F., Nelson,J.A., and Picker,L.J. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med *202*, 673-685.

Szczepura,A., Westmoreland,D., Vinogradova,Y., Fox,J., and Clark,M. (2006). Evaluation of molecular techniques in prediction and diagnosis of cytomegalovirus disease in immunocompromised patients
1. Health Technol. Assess. *10*, 1-176.

Takenaka,K., Gondo,H., Tanimoto,K., Nagafuji,K., Fujisaki,T., Mizuno,S., Miyamoto,T., Okamura,T., Hayashi,S., Eto,T., Osaki,K., Yamasaki,K., Shibuya,T., Harada,N., Teshima,T., Matsuishi,E., Minematsu,T., Minamishima,Y., Harada,M., and Niho,Y. (1997). Increased incidence of cytomegalovirus (CMV) infection and CMV-associated disease after allogeneic bone marrow transplantation from unrelated donors. The Fukuoka Bone Marrow Transplantation Group. Bone Marrow Transplant. *19*, 241-248.

Takeuchi,J., Kyo,T., Naito,K., Sao,H., Takahashi,M., Miyawaki,S., Kuriyama,K., Ohtake,S., Yagasaki,F., Murakami,H., Asou,N., Ino,T., Okamoto,T., Usui,N., Nishimura,M., Shinagawa,K., Fukushima,T., Taguchi,H., Morii,T., Mizuta,S., Akiyama,H., Nakamura,Y., Ohshima,T., and Ohno,R. (2002). Induction therapy by frequent administration of doxorubicin with four other drugs, followed by intensive consolidation and maintenance therapy for adult acute lymphoblastic leukemia: the JALSG-ALL93 study. Leukemia *16*, 1259-1266.

Talarico,C.L., Burnette,T.C., Miller,W.H., Smith,S.L., Davis,M.G., Stanat,S.C., Ng,T.I., He,Z., Coen,D.M., Roizman,B., and Biron,K.K. (1999). Acyclovir is phosphorylated by the human cytomegalovirus UL97 protein. Antimicrob. Agents Chemother. *43*, 1941-1946.

Taylor-Wiedeman,J., Sissons,P., and Sinclair,J. (1994). Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. J Virol *68*, 1597-1604.

Thiebaut,A., Vernant,J.P., Degos,L., Huguet,F.R., Reiffers,J., Sebban,C., Lepage,E., Thomas,X., and Fiere,D. (2000). Adult acute lymphocytic leukemia study testing chemotherapy and autologous and allogeneic transplantation. A follow-up report of the French protocol LALA 87. Hematol. Oncol. Clin. North Am. *14*, 1353-66, x.

Thomas,E., Storb,R., Clift,R.A., Fefer,A., Johnson,F.L., Neiman,P.E., Lerner,K.G., Glucksberg,H., and Buckner,C.D. (1975a). Bone-marrow transplantation (first of two parts). N. Engl. J. Med *292*, 832-843.

Thomas,E.D., Buckner,C.D., Banaji,M., Clift,R.A., Fefer,A., Flournoy,N., Goodell,B.W., Hickman,R.O., Lerner,K.G., Neiman,P.E., Sale,G.E., Sanders,J.E., Singer,J., Stevens,M., Storb,R., and Weiden,P.L. (1977). One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood *49*, 511-533.

Thomas,E.D., Clift,R.A., Fefer,A., Appelbaum,F.R., Beatty,P., Bensinger,W.I., Buckner,C.D., Cheever,M.A., Deeg,H.J., Doney,K., and . (1986). Marrow transplantation for the treatment of chronic myelogenous leukemia. Ann. Intern. Med. *104*, 155-163.

Thomas,E.D., Clift,R.A., Hersman,J., Sanders,J.E., Stewart,P., Buckner,C.D., Fefer,A., McGuffin,R., Smith,J.W., and Storb,R. (1982). Marrow transplantation for acute nonlymphoblastic leukemic in first remission using fractionated or single-dose irradiation. Int. J. Radiat. Oncol. Biol. Phys. *8*, 817-821.

Thomas,E.D. and Ferrebee,J.W. (1962). Prolonged storage of marow and its se in the treatment of radiation injury. Transfusion *2*, 115-117.

Thomas,E.D., Lochte,H.J.Jr., Cannon,J.H., Sahler,O.D., and Ferrebee,J.W. (1959). Supralethal whole body irradiation and isologous marrow transplantation in man. J. Clin Invest *38*, 1709-1716.

Thomas,E.D., Lochte,H.L., Jr., Lu,W.C., and Ferrebee,J.W. (1957). Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N. Engl. J. Med *257*, 491-496.

Thomas,E.D., Storb,R., Clift,R.A., Fefer,A., Johnson,L., Neiman,P.E., Lerner,K.G., Glucksberg,H., and Buckner,C.D. (1975b). Bone-marrow transplantation (second of two parts). N. Engl. J. Med *292*, 895-902.

Tomasec,P., Braud,V.M., Rickards,C., Powell,M.B., McSharry,B.P., Gadola,S., Cerundolo,V., Borysiewicz,L.K., McMichael,A.J., and Wilkinson,G.W. (2000). Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science *287*, 1031.

Tomasec,P., Wang,E.C., Davison,A.J., Vojtesek,B., Armstrong,M., Griffin,C., McSharry,B.P., Morris,R.J., Llewellyn-Lacey,S., Rickards,C., Nomoto,A.,

Sinzger,C., and Wilkinson,G.W. (2005). Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat. Immunol. *6*, 181-188.

Tomazin,R., Boname,J., Hegde,N.R., Lewinsohn,D.M., Altschuler,Y., Jones,T.R., Cresswell,P., Nelson,J.A., Riddell,S.R., and Johnson,D.C. (1999). Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. Nat. Med *5*, 1039-1043.

Tong,J.C., Zhang,G.L., Tan,T.W., August,J.T., Brusic,V., and Ranganathan,S. (2006). Prediction of HLA-DQ3.2beta ligands: evidence of multiple registers in class II binding peptides. Bioinformatics. *22*, 1232-1238.

Torok-Storb,B., Simmons,P., Khaira,D., Stachel,D., and Myerson,D. (1992). Cytomegalovirus and marrow function. Ann. Hematol. *64 Suppl*, A128-A131.

Toupance,O., Bouedjoro-Camus,M.C., Carquin,J., Novella,J.L., Lavaud,S., Wynckel,A., Jolly,D., and Chanard,J. (2000). Cytomegalovirus-related disease and risk of acute rejection in renal transplant recipients: a cohort study with case-control analyses. Transpl. Int. *13*, 413-419.

Trivedi,D., Williams,R.Y., O'Reilly,R.J., and Koehne,G. (2005). Generation of CMV-specific T lymphocytes using protein-spanning pools of pp65-derived overlapping pentadecapeptides for adoptive immunotherapy. Blood *105*, 2793-2801.

Trzonkowski,P., Mysliwska,J., Szmit,E., Wieckiewicz,J., Lukaszuk,K., Brydak,L.B., Machala,M., and Mysliwski,A. (2003). Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence. Vaccine *21*, 3826-3836.

Tsai,T., Goodman,S., Saez,R., Schiller,G., Adkins,D., Callander,N., Wolff,S., and Freytes,C.O. (1997). Allogeneic bone marrow transplantation in patients who relapse after autologous transplantation. Bone Marrow Transplant. *20*, 859-863.

Tugizov,S., Navarro,D., Paz,P., Wang,Y., Qadri,I., and Pereira,L. (1994). Function of human cytomegalovirus glycoprotein B: syncytium formation in cells constitutively expressing gB is blocked by virus-neutralizing antibodies. Virology *201*, 263-276.

Turcotte,S., Letellier,J., and Lippe,R. (2005). Herpes simplex virus type 1 capsids transit by the trans-Golgi network, where viral glycoproteins accumulate independently of capsid egress. J Virol *79*, 8847-8860.

Tyms,A.S., Scamans,E.M., and Naim,H.M. (1981). The in vitro activity of acyclovir and related compounds against cytomegalovirus infections. J. Antimicrob. Chemother. *8*, 65-72.

Ulbrecht,M., Martinozzi,S., Grzeschik,M., Hengel,H., Ellwart,J.W., Pla,M., and Weiss,E.H. (2000). Cutting edge: the human cytomegalovirus UL40 gene

product contains a ligand for HLA-E and prevents NK cell-mediated lysis. J Immunol. *164*, 5019-5022.

Vales-Gomez,M., Winterhalter,A., Roda-Navarro,P., Zimmermann,A., Boyle,L., Hengel,H., Brooks,A., and Reyburn,H.T. (2006). The human cytomegalovirus glycoprotein UL16 traffics through the plasma membrane and the nuclear envelope. Cell Microbiol. *8*, 581-590.

van der Bij,W., Torensma,R., van Son,W.J., Anema,J., Schirm,J., Tegzess,A.M., and The,T.H. (1988). Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J. Med Virol. *25*, 179-188.

van der Heiden,P.L., Kalpoe,J.S., Barge,R.M., Willemze,R., Kroes,A.C., and Schippers,E.F. (2006). Oral valganciclovir as pre-emptive therapy has similar efficacy on cytomegalovirus DNA load reduction as intravenous ganciclovir in allogeneic stem cell transplantation recipients. Bone Marrow Transplant. *37*, 693-698.

van Leeuwen,E.M., Remmerswaal,E.B., Heemskerk,M.H., ten,B., I, and van Lier,R.A. (2006). Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. Blood *108*, 3121-3127.

van Leeuwen,E.M., Remmerswaal,E.B., Vossen,M.T., Rowshani,A.T., Wertheim-van Dillen,P.M., van Lier,R.A., and ten,B., I (2004). Emergence of a CD4+. J. Immunol. *173*, 1834-1841.

van Rhee,F., Savage,D., Blackwell,J., Orchard,K., Dazzi,F., Lin,F., Chase,A., Bungey,J., Cross,N.C., Apperley,J., Szydlo,R., and Goldman,J.M. (1998). Adoptive immunotherapy for relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: equal efficacy of lymphocytes from sibling and matched unrelated donors. Bone Marrow Transplant. *21*, 1055-1061.

van Rood,J.J. and van Leeuwen,A. (1963). Leukocyte grouping. A method and its application. J. Clin Invest *42*, 1382-1390.

Verdonck,L.F., de Graan-Hentzen,Y.C., Dekker,A.W., Mudde,G.C., and de Gast,G.C. (1987). Cytomegalovirus seronegative platelets and leukocyte-poor red blood cells from random donors can prevent primary cytomegalovirus infection after bone marrow transplantation. Bone Marrow Transplant. *2*, 73-78.

Verdonck,L.F., Dekker,A.W., Lokhorst,H.M., Petersen,E.J., and Nieuwenhuis,H.K. (1997). Allogeneic versus autologous bone marrow transplantation for refractory and recurrent low-grade non-Hodgkin's lymphoma. Blood *90*, 4201-4205.

Verheyden,J.P. (1988). Evolution of therapy for cytomegalovirus infection 17. Rev. Infect. Dis. *10 Suppl 3*, S477-S489.

Verkruyse,L.A., Storch,G.A., Devine,S.M., Dipersio,J.F., and Vij,R. (2006). Once daily ganciclovir as initial pre-emptive therapy delayed until threshold

CMV load > or =10000 copies/ml: a safe and effective strategy for allogeneic stem cell transplant patients. Bone Marrow Transplant. *37*, 51-56.

Verma,A., Devine,S., Morrow,M., Chen,Y.H., Mihalov,M., Peace,D., Stock,W., Pursell,K., Wickrema,A., Yassine,M., Jessop,E., and van,B.K. (2003). Low incidence of CMV viremia and disease after allogeneic peripheral blood stem cell transplantation. Role of pretransplant ganciclovir and post-transplant acyclovir. Bone Marrow Transplant. *31*, 813-816.

Vermylen,C., Cornu,G., Ferster,A., Brichard,B., Ninane,J., Ferrant,A., Zenebergh,A., Maes,P., Dhooge,C., Benoit,Y., Beguin,Y., Dresse,M.F., and Sariban,E. (1998). Haematopoietic stem cell transplantation for sickle cell anaemia: the first 50 patients transplanted in Belgium. Bone Marrow Transplant. *22*, 1-6.

Verreck,F.A., van de,P.A., Drijfhout,J.W., Amons,R., Coligan,J.E., and Konig,F. (1996). Natural peptides isolated from Gly86/Val86-containing variants of HLA-DR1, -DR11, -DR13, and -DR52
2. Immunogenetics *43*, 392-397.

Vij,R., Khoury,H., Brown,R., Goodnough,L.T., Devine,S.M., Blum,W., Adkins,D., and Dipersio,J.F. (2003). Low-dose short-course intravenous ganciclovir as pre-emptive therapy for CMV viremia post allo-PBSC transplantation. Bone Marrow Transplant. *32*, 703-707.

Villacres,M.C., Lacey,S.F., Auge,C., Longmate,J., Leedom,J.M., and Diamond,D.J. (2003). Relevance of peptide avidity to the T cell receptor for cytomegalovirus-specific ex vivo CD8 T cell cytotoxicity. J. Infect. Dis. *188*, 908-918.

Vlieger,A.M., Boland,G.J., Jiwa,N.M., de Weger,R.A., Willemze,R., de Gast,G.C., and Falkenburg,J.H. (1992). Cytomegalovirus antigenemia assay or PCR can be used to monitor ganciclovir treatment in bone marrow transplant recipients. Bone Marrow Transplant. *9*, 247-253.

von Dadelszen,P., Magee,L.A., Krajden,M., Alasaly,K., Popovska,V., Devarakonda,R.M., Money,D.M., Patrick,D.M., and Brunham,R.C. (2003). Levels of antibodies against cytomegalovirus and Chlamydophila pneumoniae are increased in early onset pre-eclampsia. BJOG. *110*, 725-730.

Vusirikala,M., Wolff,S.N., Stein,R.S., Brandt,S.J., Morgan,D.S., Greer,J.P., Schuening,F.G., Dummer,J.S., and Goodman,S.A. (2001). Valacyclovir for the prevention of cytomegalovirus infection after allogeneic stem cell transplantation: a single institution retrospective cohort analysis. Bone Marrow Transplant. *28*, 265-270.

Wagner,J.E., Donnenberg,A.D., Noga,S.J., Cremo,C.A., Gao,I.K., Yin,H.J., Vogelsang,G.B., Rowley,S., Saral,R., and Santos,G.W. (1988). Lymphocyte depletion of donor bone marrow by counterflow centrifugal elutriation: results of a phase I clinical trial. Blood *72*, 1168-1176.

Waldmann,H., Polliak,A., Hale,G., Or,R., Cividalli,G., Weiss,L., Weshler,Z., Samuel,S., Manor,D., Brautbar,C., and . (1984). Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet *2*, 483-486.

Waldrop,S.L., Davis,K.A., Maino,V.C., and Picker,L.J. (1998). Normal human CD4+ memory T cells display broad heterogeneity in their activation threshold for cytokine synthesis. J. Immunol. *161*, 5284-5295.

Waldrop,S.L., Pitcher,C.J., Peterson,D.M., Maino,V.C., and Picker,L.J. (1997). Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J. Clin. Invest *99*, 1739-1750.

Walter,E.A., Greenberg,P.D., Gilbert,M.J., Finch,R.J., Watanabe,K.S., Thomas,E.D., and Riddell,S.R. (1995). Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N. Engl. J. Med *333*, 1038-1044.

Walters,M.C., Storb,R., Patience,M., Leisenring,W., Taylor,T., Sanders,J.E., Buchanan,G.E., Rogers,Z.R., Dinndorf,P., Davies,S.C., Roberts,I.A., Dickerhoff,R., Yeager,A.M., Hsu,L., Kurtzberg,J., Ohene-Frempong,K., Bunin,N., Bernaudin,F., Wong,W.Y., Scott,J.P., Margolis,D., Vichinsky,E., Wall,D.A., Wayne,A.S., Pegelow,C., Redding-Lallinger,R., Wiley,J., Klemperer,M., Mentzer,W.C., Smith,F.O., and Sullivan,K.M. (2000). Impact of bone marrow transplantation for symptomatic sickle cell disease: an interim report. Multicenter investigation of bone marrow transplantation for sickle cell disease. Blood *95*, 1918-1924.

Wang,X., Huang,D.Y., Huong,S.M., and Huang,E.S. (2005). Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. Nat. Med *11*, 515-521.

Wang,X., Huong,S.M., Chiu,M.L., Raab-Traub,N., and Huang,E.S. (2003). Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature *424*, 456-461.

Weekes,M.P., Wills,M.R., Mynard,K., Carmichael,A.J., and Sissons,J.G. (1999). The memory cytotoxic T-lymphocyte (CTL) response to human cytomegalovirus infection contains individual peptide-specific CTL clones that have undergone extensive expansion in vivo. J. Virol. *73*, 2099-2108.

Weekes,M.P., Wills,M.R., Sissons,J.G., and Carmichael,A.J. (2004). Long-term stable expanded human CD4+ T cell clones specific for human cytomegalovirus are distributed in both CD45RAhigh and CD45ROhigh populations. J. Immunol. *173*, 5843-5851.

Weiden,P.L., Flournoy,N., Thomas,E.D., Prentice,R., Fefer,A., Buckner,C.D., and Storb,R. (1979). Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N. Engl. J. Med *300*, 1068-1073.

Weill,D., Lock,B.J., Wewers,D.L., Young,K.R., Zorn,G.L., Early,L., Kirklin,J.K., and McGiffin,D.C. (2003). Combination prophylaxis with ganciclovir and cytomegalovirus (CMV) immune globulin after lung transplantation: effective CMV prevention following daclizumab induction. Am J Transplant. *3*, 492-496.

Westphal,M., Lautenschlager,I., Backhaus,C., Loginov,R., Kundt,G., Oberender,H., Stamm,C., and Steinhoff,G. (2006). Cytomegalovirus and proliferative signals in the vascular wall of CABG patients. Thorac. Cardiovasc. Surg. *54*, 219-226.

Wikby,A., Johansson,B., Olsson,J., Lofgren,S., Nilsson,B.O., and Ferguson,F. (2002). Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Exp Gerontol. *37*, 445-453.

Willemze,R., Richel,D.J., Falkenburg,J.H., Hale,G., Waldmann,H., Zwaan,F.E., and Fibbe,W.E. (1992). In vivo use of Campath-1G to prevent graft-versus-host disease and graft rejection after bone marrow transplantation. Bone Marrow Transplant. *9*, 255-261.

Williams,R.J., Clarke,E., Blair,A., Evely,R., Hale,G., Waldmann,H., Brookes,S., and Pamphilon,D.H. (2000). Impact on T-cell depletion and CD34+ cell recovery using humanised CD52 monoclonal antibody (CAMPATH-1H) in BM and PSBC collections; comparison with CAMPATH-1M and CAMPATH-1G. Cytotherapy. *2*, 5-14.

Wills,M.R., Ashiru,O., Reeves,M.B., Okecha,G., Trowsdale,J., Tomasec,P., Wilkinson,G.W., Sinclair,J., and Sissons,J.G. (2005). Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis. J. Immunol. *175*, 7457-7465.

Wills,M.R., Carmichael,A.J., Mynard,K., Jin,X., Weekes,M.P., Plachter,B., and Sissons,J.G. (1996). The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J. Virol. *70*, 7569-7579.

Wingard,J.R., Mellits,E.D., Sostrin,M.B., Chen,D.Y., Burns,W.H., Santos,G.W., Vriesendorp,H.M., Beschorner,W.E., and Saral,R. (1988). Interstitial pneumonitis after allogeneic bone marrow transplantation. Nine-year experience at a single institution. Medicine (Baltimore) *67*, 175-186.

Winston,D.J., Ho,W.G., Bartoni,K., Du,M.C., Ebeling,D.F., Buhles,W.C., and Champlin,R.E. (1993). Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. Ann Intern Med *118*, 179-184.

Winston,D.J., Huang,E.S., Miller,M.J., Lin,C.H., Ho,W.G., Gale,R.P., and Champlin,R.E. (1985). Molecular epidemiology of cytomegalovirus infections associated with bone marrow transplantation. Ann Intern Med *102*, 16-20.

Winston,D.J., Yeager,A.M., Chandrasekar,P.H., Snydman,D.R., Petersen,F.B., and Territo,M.C. (2003). Randomized comparison of oral valacyclovir and intravenous ganciclovir for prevention of cytomegalovirus disease after allogeneic bone marrow transplantation. Clin Infect. Dis. *36*, 749-758.

Xhaard,A., Robin,M., Scieux,C., de Latour,R.P., Deplus,S., Mazeron,M.C., Devergie,A., Esperou,H., Rocha,V., Gluckman,E., Ribaud,P., and Socie,G. (2007). Increased incidence of cytomegalovirus retinitis after allogeneic hematopoietic stem cell transplantation
2. Transplantation *83*, 80-83.

Xiao,Y. and Segal,M.R. (2005). Prediction of genomewide conserved epitope profiles of HIV-1: classifier choice and peptide representation. Stat. Appl. Genet. Mol. Biol. *4*, Article25.

Yanada,M., Yamamoto,K., Emi,N., Naoe,T., Suzuki,R., Taji,H., Iida,H., Shimokawa,T., Kohno,A., Mizuta,S., Maruyama,F., Wakita,A., Kitaori,K., Yano,K., Hamaguchi,M., Hamajima,N., Morishima,Y., Kodera,Y., Sao,H., and Morishita,Y. (2003). Cytomegalovirus antigenemia and outcome of patients treated with pre-emptive ganciclovir: retrospective analysis of 241 consecutive patients undergoing allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant. *32*, 801-807.

Yates,P.J. and Nicholson,M.L. (2006). The aetiology and pathogenesis of chronic allograft nephropathy. Transpl. Immunol. *16*, 148-157.

Yeager,A.M. (2002). Allogeneic hematopoietic cell transplantation for inborn metabolic diseases
9. Ann. Hematol. *81 Suppl 2*, S16-S19.

Yeager,A.S., Grumet,F.C., Hafleigh,E.B., Arvin,A.M., Bradley,J.S., and Prober,C.G. (1981). Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J. Pediatr. *98*, 281-287.

Yoshihara,T., Morimoto,A., Inukai,T., Kuroda,H., Ishida,H., Sugita,K., Goi,K., Imamura,T., Todo,S., Maruya,E., Saji,H., Nakazawa,S., and Imashuku,S. (2004). Non-T-cell-depleted HLA haploidentical stem cell transplantation based on feto-maternal microchimerism in pediatric patients with advanced malignancies. Bone Marrow Transplant. *34*, 373-375.

Zanghellini,F., Boppana,S.B., Emery,V.C., Griffiths,P.D., and Pass,R.F. (1999). Asymptomatic primary cytomegalovirus infection: virologic and immunologic features. J. Infect. Dis. *180*, 702-707.

Zhu,H., Shen,Y., and Shenk,T. (1995). Human cytomegalovirus IE1 and IE2 proteins block apoptosis
1. J. Virol. *69*, 7960-7970.

Zimmerli,W., Zarth,A., Gratwohl,A., and Speck,B. (1991). Neutrophil function and pyogenic infections in bone marrow transplant recipients
23. Blood *77*, 393-399.

Appendix A

Patient Consent for HCMV Immune Reconstitution Study

PARTICIPANT CONSENT FORM

TITLE OF PROJECT

Monitoring of CMV antigen specific T cells in Bone marrow transplant patients before and after transplant.

The patient should complete the whole of this sheet themselves.

Have you read the Patient Information Sheets? **YES / NO**

Have you had an opportunity to ask questions and discuss this study? **YES / NO**

Have you received satisfactory answers to all your questions? **YES / NO**

Have you received enough information about the study? **YES / NO**

Who have you spoken to? Dr / Mr / Mrs_____

Do you understand that you are free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting your future medical care? **YES / NO**

Do you agree to enter your this study and in doing so give permission for the researchers to have access to your medical notes? **YES / NO**

Do you give permission for us to notify your GP that you have entered this study? **YES / NO**

Do you agree to enter in this study? **YES / NO**

Patient's name _____
(in block letters)

Signature _____ Date |___|___|___|

Dr's signature _____ Date |___|___|___|

Dr's name _____
(in block letters)

File one copy in patients notes, one copy in CRF and one copy to patient.

Patient Information Sheet

Monitoring of CMV antigen specific T cells in Bone Marrow transplantpatiens before and after transplant.

We at the Anthony Nolan Research Institute and Haematology Dept of the RFH are currently embarking on a project to investigate the reconstitution of the immune system after bone marrow transplantation. As you are aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections but of specific importance to transplant patients is CMV. The precise details of how fast these cells regenerate and how effective they are in fighting CMV virus in Bone marrow transplant patients still remains unclear. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood. These samples would be taken at the same time as the regular checks occurring at the hospital and will not require additional venepuncture.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and then subsequently at the same times when blood is being taken for routine monitoring of CMV status, this is usually twice weekly during the early phase post transplant. Monitoring will continue at outpatient appointments.

DONORS- blood is required once at the pre-donation health check.

The cells extracted from these samples will only be used for the research project that the patients has agreed to take part in.

The proposed study will use class I soluble HLA molecules in the form of tetramers. These will be used to specifically detect the presence of circulating antigen specific T cells in the peripheral blood of patients who reactivate CMV following BMT.
CMV viraemia is a significant problem in immunosuppressed patients following BMT and before their new haemopoietic system has been able to fully develop to provide appropriate level of immune response to counteract the occurrence of CMV infection de novo or through the reactivation of pre-existing CMV. Monitoring the immune potential of a patient to mount a specific immune response to a virus such as CMV, will allow us to obtain a better understanding of what is happening to the CMV specific T cells in patients who reactivate CMV post BMT or who develop de-novo CMV viraemia following BMT. The acquisition of such data will allow us to correlate the ability of patients to resolve CMV with the concurrent expansion or development of CMV specific CD8 T cells in their peripheral circulation. The analysis of such imformation has implications for improved therapy or clinical intervention at specific time points resulting in improved treatment of patients developing CMV disease post BMT.

The role of CMV specific CD8+ T cells in the resolution of CMV viraemia is well documented. However specific details about the dynamics of expanded CMV +ve populations, the relative relecance of CD4+ T cells to the action of CD8 antigen.

Appendix B

pp65 Peptide Pool Composition

Peptide#	*Sequence*	
1	MESRGRCPEMISVL	
2	GRRCPEMISVLGPIS	
3	PEMISVLGPISGHVL	
4	SVLGPISGHVLKAVF	
5	PISGHVLKAVFSRGD	
6	HVLKAVFSRGDTPVL	
7	AVFSRGDTPVLPHET	
8	RGDTPVLPHETRLLQ	Pool 1
9	PVLPHETRLIQTGIH	
10	HETRLLQTGIHVRVS	
11	LLQTGIHVRVSQPSL	
12	GIHVRVSQPSLILVS	
13	RVSQPSLILVSQYTP	
14	PSLILVSQYTPDSTP	
15	LVSQYTPDSTPCHRG	
16	YTPDSTPCHRGDNQL	
17	STPCHRGDNQLQVQH	
18	HRGDNQLQVQHTYFT	
19	NQLQVQHTYFTGSEV	
20	VQHTYFTGSEVENVS	
21	YFTGSEVENVSVNVH	
22	SEVENVSVNVHNPTG	
23	NVSVNVHNPTGRSIC	Pool 2
24	NVHNPTGRSICPSQE	
25	PTGRSICPSQEPMSI	
26	SICPSQEPMSIYVYA	
27	SQEPMSIYVYALPLK	
28	MSIYVYALPLKMLNI	
29	VYALPLKMLNIPSIN	
30	PLKMLNIPSINVHHY	
31	LNIPSINVHHYPSAA	
32	SINVHHYPSAAERKH	
33	HHYPSAAERKHRHLP	
34	SAAERKHRHLPVADA	
35	RKHRHLPVADAVIHA	
36	HLPVADAVIHHASGKQ	
37	ADAVIHASGKQMWQA	
38	IHASGKQMWQARLTV	Pool 3
39	GKQMWQARLTVSGLA	
40	WQARLTVSGLAWTRQ	
41	LTVSGLAWTRQQNQW	
42	GLAWTRQQNQWKEPD	
43	TRQQNQWKEPDVYYT	
44	NQWKEPDVYYTSAFV	
45	EPDVYYTSAFVFPTK	
46	YYTSAFVFPTKDVAL	

47	AFVFPTKDVALRHVV	
48	PTKDVALRHVVCAHE	
49	VALRHVVCAHELVCS	
50	HVVCAHELVCSMENT	
51	AHELVCSMENTRATK	
52	VCSMENTRATKMQVI	
53	ENTRATKMQVIGDQY	Pool 4
54	ATMQVIGDQYVKVY	
55	QVIGDQYVKVYLESF	
56	DQYVKVYLESFCEDV	
57	KVYLESFCEDVPSGK	
58	ESCEDVPSGKLFMH	
59	EDVPSGKLFMHVTLG	
60	SGKLFMHVTLGSDVE	
61	FMHVTLGSDVEEDLT	
62	TLGSDVEEDLTMTRN	
63	DVEEDLTMTRNPQPF	
64	DLTMTRNPQPFMRPH	
65	TRNPQPFMRPHERNG	
66	QPFMRPHERNGFTVL	
67	RPHERNGFTVLVPKN	Pool 5
68	RNGFTVLCPKNMIIK	
69	TVLCPKNMIIKPGKI	
70	PKNMIIKPGKISHIM	
71	IIKPGKISHIMLDVA	
72	GKISHIMLDVAFTSH	
73	HIMLDVAFTSHEHFG	
74	DVAFTSHEHFGLICP	
75	TSHEHFGLICPKSIP	
76	HFGLLCPKSIPGLSI	
77	LCPKSIPGLSISGNL	
78	SIPGLSISGNLIMNG	
79	LSISGNLLMNGQQIF	
80	GNLLMNGQQIFLEVQ	
81	MNGQQIFLEVQAIRE	
82	QIFLEVQAIRETVEL	
83	EVQAIRETVELRQYD	Pool 6
84	IRETVELRQYDPVAA	
85	VELRQYDPVAALFFF	
86	QYDPVAALFFFDIDL	
87	VALFFFDIDLLLQR	
88	FFFDIDLLLQRGPQY	
89	IDLLLQRGPQYSEHP	
90	LQRGPQYSEHPTFTS	
91	PQYSEHPTFTSQYRI	
92	EHPTFTSQYRIQGKL	
93	FTSQYRIQGKLEYRH	
94	YRIQGKLEYRHTWDR	
95	GKLEYRHTWDRHDEG	
96	YRHTWDRHDEGAAQG	
97	WDRHDEGAAQGDDDV	
98	DEGAAQGDDDVWTSG	Pool 7
99	AQGDDDVWTSGSDSD	

100	DDVWTSGSDSDEELV	
101	TSGDSDEELVTTER	
102	DSDEELVTTERKTPR	
103	ELVTTERKTPRVTGG	
104	TERKTPRVTGGGAMA	
105	TPRVTGGGAMAGAST	
106	TGGGAMAGASTSAGR	
107	AMAGASTSAGRKRKS	
108	ASTSAGRKRKSASSA	
109	AGRKRKSASSATACT	
110	RKSASSATACTSGVM	
111	SSATACTSGVMTRGR	
112	ACTSGVMTRGRLKAE	
113	GVMTRGRLKAESTVA	Pool 8
114	RGRLKAESTVAPEED	
115	KAESTVAPEEDTDED	
116	TVAPEEDTDEDSDNE	
117	EEDTEDSDNEIHNP	
118	DEDSDNEIHNPAVFT	
119	DNEIHNPAVFTWPPW	
120	HNPAVFTWPPWQAGI	
121	VFTWPPWQAGILARN	
122	PPWQAGILARNLVPM	
123	AGILARNLVPMVATV	
124	ARNILPMVATVQGQN	
125	VPMVATVQGQNLKYQ	
126	ATVQGQNLKYQEFFW	
127	GQNLKYQEFFWDAND	
128	KYQEFFWDANDIYRI	Pool 9
129	FFWDANDIYRIFAEL	
130	ANDIYRIFAELEGVW	
131	YRIFAELEGVWQPAA	
132	AELEGVWQPAAQPKR	
133	GVWQPAAQPKRRRHR	
134	PAAQPKRRRHRQDAL	
135	PKRRRHRQDALPGPC	
136	RHRQDALPGPCIAST	
137	DALPGPCIASTPKKH	
138	LPGPCIASTPKKHRG	

IE1 Peptide Pool Composition

Peptide#	*Sequence*	
1	MESSAKRKMDPDNPD	
2	AKRKMDPDNPDEGPS	
3	MDPDNPDEGPSSKVP	
4	NPDEGPSSKVPRPET	
5	GPSSKVPRPETPVTK	
6	KVPRPETPVTKATTF	
7	PETPVTKATTFLQTM	
8	VTKATTFLQTMLRKE	Pool 1
9	TTFLQTMLRKEVNSQ	
10	QTMLRKEVNSQLSLG	
11	RKEVNSQLSLGDPLF	
12	NSQLSLGDPLFPFPELA	
13	SLGDPLFPELAEESL	
14	PLFPELAEESLKTFE	
15	ELAEESLKTFEQVTE	
16	ESLKTFEQVTEDCNE	
17	TFEQVTEDCNENPEK	
18	VTEDCNENPEKDVLA	
19	CNENPEKDVLAELVK	
20	PEKDVLAELVKQIKV	
21	VLAELVKQIKVRVDM	
22	LVKQIKVRVDMVRHR	
23	IKVRVDMVRHRIKEH	Pool 2
24	VDMVRHRIKEHMLKK	
25	RHRIKEHMLKKYTQT	
26	KEHMLKKYTQTEEKF	
27	LKKYTQTEEKFTGAF	
28	TQTEEKFTGAFNMMG	
29	EKFTGAFNMMGGCLQ	
30	GAFNMMGGCLQNALD	
31	MMGGCLQNALDILDK	
32	CLQNALDILDKVHEP	
33	ALDIDKVHEPFEEM	
34	LDKVHEPFEEMKCIG	
35	HEPFEEMKCIGLTMQ	
36	EEMKCIGLTMQSMYE	
37	CIGLTMQSMYENYIV	
38	TMQSMYENYIVPEDK	Pool 3
39	MYENYIVPEDKREMW	
40	YIVPEDKREMWMACI	
41	EDKREMWMACIKELH	
42	EMWMACIKELHDVSK	
43	ACIKELHDVSKGAAN	
44	ELHDVSKGAANKLGG	
45	VSKGAANKLGGALQA	
46	AANKLGGALQAKARA	
47	LGGALQAKARAKKDE	
48	LQAKARAKKDELRRK	

49	ARAKKDELRRKMMYM	
50	KDELRRKMMYMCYRN	
51	RRKMMYMCYRNIEFF	
52	MYMCYRNIEFFTKNS	
53	YRNIEFFTKNSAFPK	Pool 4
54	EFFTKNSAFPKTTNG	
55	KNSAFPKTTNGCSQA	
56	FPKTTNGCSQAMAAL	
57	TNGCSQAMAALQNLP	
58	SQAMAALQNLPQCSP	
59	AALQNLPQCSPDEIM	
60	NLPQCSPDEOMAYAQ	
61	CSPDEIMAYAQKIFK	
62	EIMAYAQKIFKILDE	
63	YAQKIFKILDEERKDK	
64	IFKILDEERDKVLTH	
65	LDEERDKVLTHIDHI	
66	RDKVLTHIDHIFMDI	
67	LTHIDHIFMDILTTC	Pool 5
68	DHIFMDILTTCVETM	
69	MDILTTCVETMCNEY	
70	TTCVETMCNEYKVTS	
71	ETMCNEYKVTSDACM	
72	NEYKVTSDACMMTMY	
73	VTSDACMMTMYGGIS	
74	ACMMTMYGGISLLSE	
75	TMYGGISLLSEFCRV	
76	GISLLSEFCRVLCCY	
77	LSEFCRVLCCYVLEE	
78	CRVLCCYVLEETSVM	
79	CCYVLEETSVMLAKR	
80	LEETSVMLAKRPLIT	
81	SVMLAKRPLITKPEV	
82	AKRPLITKPEVISVM	
83	LITKPEVISVMKRRI	Pool 6
84	PEVISVMKRRIEEIC	
85	SVMKRRIEEICMKVF	
86	RRIEEICMKVFAQYI	
87	EICMKVFAQYILGAD	
88	KVFAQYILGADPLRV	
89	QYILGADPLRVCSPS	
90	GADPLRVCSPSVDDL	

91	LRVCSPSVDDLRAIA	
92	SPSVDLRAIAEESD	
93	DDLRAIAEESDEEEA	
94	AIAEESDEEEAIVAY	
95	ESDEEEAIVAYTLAT	
96	EEAIVAYTLATAGVS	
97	VAYTLATAGVSSSDS	
98	LATAGVSSSDSLVSP	Pool 7
99	GVSSSDSLSPPESP	
100	SDSLVSPPESPVPAT	
101	VSPPESPVPATIPLS	
102	ESPVPATIPLSSVIV	
103	PATIPLSSVIVAENS	
104	PLSSVIVAENSDQEE	
105	VIVAENSDQEESEQS	
106	ENSDQEESEQSDEEE	
107	QEESEQSDEEEEEGA	
108	EQSDEEEEEGAQEER	
109	EEEEEGAQEEREDTV	
110	EGAQEEREDTVSVKS	
111	EEREDTVSVKSEPVS	
112	DTVSVKSEPVSEIEE	
113	VKSEPVSEIEEVAPE	Pool 8
114	PVSEIEEVAPEEEED	
115	IEEVAPEEEEDGAEE	
116	APEEEEDGAEEPTAS	
117	EEDGAEEPTASGGKS	
118	AEEPTASGGKSTHPM	
119	TASGGKSTHPMVTRS	
120	GKSTHPMVTRSKADQ	